Department Virology

Subject Vaccine Development

Name Michael Parker

Address APC M.A.I.C

Lab Notebook # 3354

National Brand

Issued 8-28-91 43-648

Computation Notebook

Dennison Stationery Products Co., Framingham, MA 01701

0 73333 43648 8

75 Sheets
11¾" x 9¼"
4x4 Quad.

EXHIBIT L

BEST AVAILABLE COPY

PE6-cDNA Synthesis	3
PE6- PCR	5
Cloning F. Strigki's PCR fragments	11
CCHF M Segment Sequencing	17
EEE, WEE cDNA Synthesis	19
Assembly of PE6 26S cDNA's	41
Mayaro Virus Preparation	47
CHIK Vaccine Parent Control	49
Hg Agarose Gels	51
Purification intracellular RNP RVF	59
PCR OF Remaining CHIK SEQUENCE	71
CHIK CLONE MAP	76 H
Preparation of 143, TK Vector	83
Replicon Helper Preparation	133
PCEV-5 SEQUENCE	139
HATCHEL VIRUS - CHIK - GROWTH	141
CLONING 1.7KB M PCR PRODUCT - CCHF	145

From 3353 p148

Ligation of Custom pCEV6 cDNA 7-3-92

Sample	Vector	cDNA	5x	H_2O	Ligase
Random cDNA	2	5/10	6		2
Oligo dT cDNA	2	5/10	6		2
Vector control	2	0	6		2

Vector = pUC13 Sma1 10ng/ml

7-21-92
Pick Ribosoft colonies from Oligo dT primed material and mini prep to identify and Containing Missing Nylon. - 12 picked by Angel.

Very few (4) white colonies obtained from Random primed material, all 4 picked for miniprep - 7/28/92

- Bam Hi cutting of Random primed cDNA
- Ethoh ppt
Dry

7-28-92
Sephacryl 300 Spin column left ~ 700cpm/µl, Random Primed cDNA less than 3% of initial. ~~Repeated~~
~~Repeat~~ - NOT USING KIT!
Fractionate and ligate!

7-31-92
RT Buffer cocktail OD260/280 RNA 1.8

50mM tris pH8.3	5µl 1M		
10mM $MgCl_2$	10µl	100mM	RNA Suspended in
10mM DTT	2µl	500mM	500µl H_2O and
70mM KCl	7µl	1M.	read directly.

- Pt'd in 10ug aliquots -

7/1/92
2µl samples

			cpm	net	%	nM	ng cDNA
X RNA - 10µl	60% Inhib						
+ Random primers	- 5µl - (2M)		✓ 0	975929			
+ dNTP - 5mM	- 10µl		✓ 0	+ 1900			
X ^{35}S - dATP	- 3µl		60	+ 74647	72747	.07	14.9 4.6ng
+ RNAsin	- 2.0µl (20u, 6180)						
+ Buffer	- 2µl						
+ RT	4µl (22.9u/µl, Life Sci.)						
+ H_2O	4µl ~~0~~ 1M						

Very High.

from page 3

✓ 8µl 500mM EDTA
✓ 100µl TE
✓ ~~0~~ 0.4 2x
✓ 133µl 5M NH_4OAc
✓ 800µl EtOH
✓ -70°C 20'
- Warm to RmT
- Pellet
- Repeat precipitation
- Wash pellet & dry.

Small fragment & pellet was lost - I think during washing

2nd strand synthesis - ^3H label

2nd Strand Buffer

10mM tris pH 7.4	1µl 1M
5mM $MgCl_2$	2.5µl 100mM
10mM $(NH_4)_2SO_4$	5µl 100mM
100mM KCl	5µl 1M
	13.5µl

26.5µl H_2O
+13.5µl buffer cocktail
2.5µl dGTP
5µl ^3H-dCTP
✓ 1µl RNase H - Boehringer Kit reagents
✓ 2.5µl Pol I -

	1st samples	
12°C - 2 hrs	1:30-3:00	♂ - 77384
22°C - 2 hrs	3-4:30	♂ + 2401
		♀ 11200

8-12-92
-4 blunt ending

What happened to Cpne?

100µl blunt ending reaction

80µl H_2O
10µl 10x T4 buffer
5µl 5mM dNTP
5µl - 5u - T4 PL - BM

37°C, 20'

to page 9

7-30-97

from 3353 page 148.

Inventory of digested EEE-1 PCR fragments

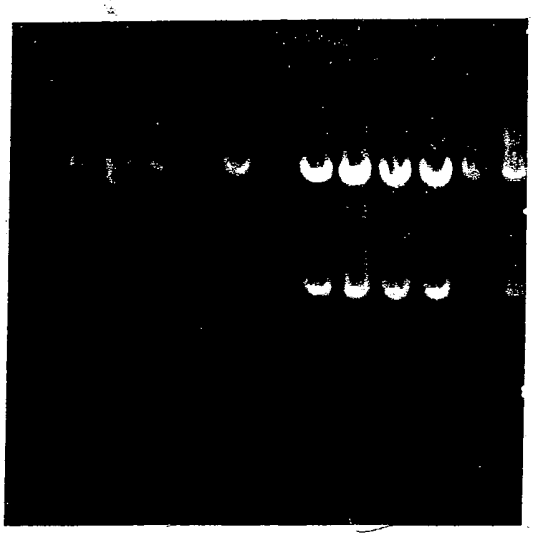
Sample	Vol/rxn	Ins	Sx	H2O	Ligase
Sal/Frag	3	5	6	15	1
Vol/rxn	3	0	6	20	1
R1/H3Frag-NOT	2	5	6	16	1
Vol/rxn	2	0	6	21	1

7-21-97

Aliquoted to distinguish 2 possible Sal clones and 6 possible ~~Bam~~/Hind3 clones.

1 possible Sal clone
5 possible H3/Bam Clones isolated

Orient Sal clone By R1 and Hind3 digestion
H3/Bam Clones by RV/Hind3 and RV/Bam digestion



Lanes 1-6
Sal Frag
+ Sac1
- Marking not visible -
7-12
R1/H3
+ R1/H3

7-30-97
Sequencing by Dan Pendleton
in E265-Sal folder

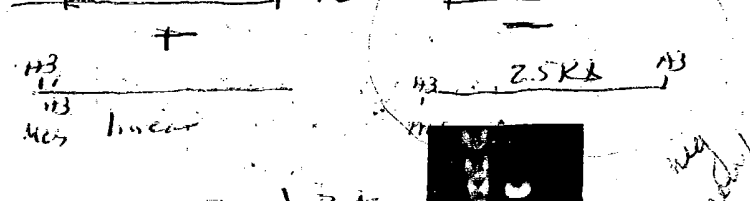
3 - mapped by R1 and H3 digestion

→ ~~EEE~~ Sal E265-Sal, Box ~~EE~~ Slot 212

7 RV/Bam, RV/H3 digested to determine orientation
also digest with Bam/H3 and compare to original frag

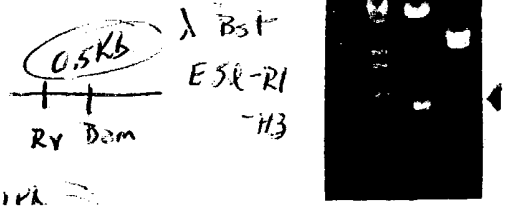
If #3 is correct in pT218Sal

R1 digestion →



H3

#7 RV/BAM in Bam/H3 pT218



Dan/H3

1.1Kb

Dan

Assembly of full length EEE-265

Sal I - Hind 3 digest EEE-Sal
Hind 3 digest - Bam Digest H3/Bam Frag

Ligate into Sal I - Bam HI pT219.
Initially digest with Sal I followed by addition of Bam HI

10 µl pT219 1.6 µg/µl
5 µl 10x Hi-fi
33 µl H_2O
2 µl Sal I
-37°C 8:30 a.m.

To Page 7

Sequence determination of missing portion of 265
Using EEE-Sal (pT218)

Digest with (Sac I or Kpn (resistant)
Ba 10:00 (susceptible)

- 20 second time pts taken, (5), 5 minute pt also
- Deletion working
- Samples 1-5 eluted, repaired & ligated 4:00
- into JM105 for ϕ preparation.
Onto X gal plates. 7-31-92

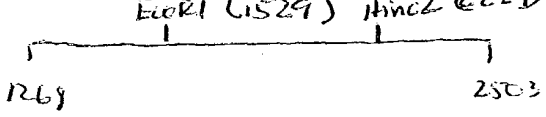
- two Colonies obtained
- Transform again with #'s 1 & 2 8-3-92
Colonies picked 8-5-92
Sequence 8-6-92

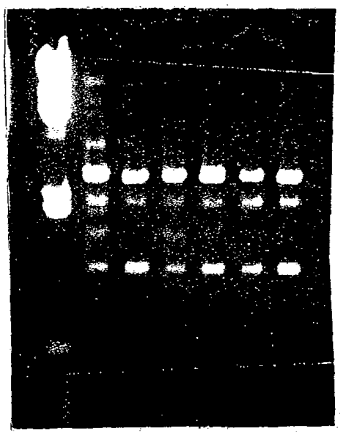
from pg 6.

E265 Sequence determination 8-2-92

Sph Fragment ~ 1.2Kb from E265-Sal Subcloned into pTZ 18.

Fragment extends from 1268 - 2503.
Six clones picked - all positive
Map by Eco RI digestion + Hinc 2 digestion



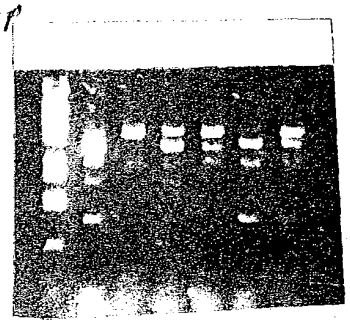


8-3-92
RI + Hinc2 digestions by Angel Sph 1 1-6

Φ cultures started for sequencing on 8-4-92
Angel did a double digest - will be repeated.

With respect to T7 promoter

5' ⟶ 3'	RI ⟶ ~1100 bp	H_2 ⟶ 300 bp
3' ⟵ 5'	RI ⟶ 300 bp	H_2 ⟶ 1000 bp



E1 + 2 2-1 to JM105's for sequencing

Frozen #1 5' ⟶ 3' slot 213 Sph 1-6
#2 3' ⟵ 5' slot 214 Eco RI

E265 - Sph #1, 2. - pHSu

Slot 215 213 in JM105
216 214 "

Prepare Φ for sequencing 8-11-92

Sequence run 8-12-92

Edited sequence files E265-213

9-5-92

cDNA cloning of pE6 - 5' extensions

10ng RNA pelleted and dried.

Buffer cocktail

50mM Tris pH 8.3	5µl	1M ✓
10mM $MgCl_2$	10µl	100mM ✓
10mM DTT	10µl	100mM ✓
70mM KCl	7µl	1M ✓

On ice

RNA in 20µl H_2O

3µl random primer

10µl 5mM dNTP

35-dATP - 3µl

2.5µl RNAsin

32µl buffer cocktail

4µl AMV RT - 100units

25.5µl H_2O

	Vol	%	nM	ug cDNA
		to		
37°C - 5'		10		
42°C - 40'		to		

2µl sample

8µl 500mM EDTA

100µl H_2O

EtOH 2x

133µl 5M NH_4OAc

800µl EtOH

Dry Ice to freeze

To Rm T

Spin

Repeat

8-12-92

- 5µl EDTA
- Phenol extraction
- S-300 Columns

1µl eluate - 12,400 cpm
100

Counts are inconsistent throughout

EtOH ppt eluate

8-12-92

Ligations -

Pellet cDOA, rinse 1 dry.

Ligate into Hind II site of pTZ18

① Suspend cDOA in 30µl H_2O

Combine Ⓐ 5µl cDOA 1µl vector (195ng/µl)
Ⓑ 5µl cDOA 1µl vector 34 ng

Equivalent controls (C,D)

Sample	Amount	Vector	10x YD	H_2O	Lig	
A	5	1	3	19	2	ⓐ
B	5	2	3	18	2	
C	—	1	3	24	2	
D	—	2	3	23	2	ⓑ
	x	x	x			

Also Talk about vector - Why so much white colony formation, Ltr to JM105.

E	—	5	3-10x YD	21	1	blue, talk
F	—	5	6- 5a BRL	18	1	~~more blue~~ call blue
		x	✓	x		

8-13-92

3µl each of A-D in Library efficiency Diff 5a

8-14-92

Lack of colonies probably due to low recovery of cDOA
- Start again.

Cloning Julie's PCR fragments. 8-13-92

x ① Extract reaction 1x w/ $CHCl_3$ ÷ EtOH ppt
-70°C, 15'

② Resuspend in 50µl H_2O

Remainder frozen at -70 "Crude PCRx")

③ Repair 25µl aliquots w/ T4 Pol
- 25µl ~~10x T4 Pol~~ Fragment
- 5µl 10x T4 Buffer
- 1µl H_2O
- 2µl - 2u - T4 Pol (Boehringer)
- 2µl - dwtp 5mM

Should have had buffer & incubation w/o dwtp present

✓ 37°C, 20
2µl 500mM EDTA
~~1µl H_2O~~
100µl ~~EtOH~~

~~- 5-300 Spun column~~
- EtOH ppt
- Pellet and dry
- Resolve on Agarose TBE · 90V 2 hrs.
- Elute w/ Gene Clean
- Ligate into pT2 18-Hinc 2 - ~~#38~~ w/ BRL ÷ YD LIGASE BUFFERS

LIGATION OLIGO MUST BE PHOSPHORYLATED

~~BRL BUFFER~~

Sample	Insert	Vector	5x	H_2O	Ligase	White	Blue
1	5	2	6	15	2	0	11
2	5	2	6	15	2	- 1	6
3	5	2	6	15	2	6	6
4	5	2	6	15	2	- 1	4
Vector	0	2	6	20	2	0	4

YD LIGASE — 10x - Red label

5 (1)	5	2	3	18	2	0	8
6 (2)	5	2	3	18	2	0	4
7 (3)	5	2	3	18	2	- 1	15
8 (4)	5	2	3	18	2	0	2
V2	0	2	3	17	2		

S-A-92

Phosphorylation of oligo's

Gene Cloned oligo's in H_2O
① Add 10x PNK buffer
② q.s. 35µl
③ Add 2µl PNK
④ 37°C 60'
⑤ 65°C 10'
6 - Ligate 5µl to Hinc 2 cut vector

10x PNK - ~~BRL~~ USB
~~700mM 660mM tris pH 7.0~~
~~MgCl~~
~~100mM MgCl2~~
~~50mM DTT~~
~~5mg/ml BSA~~
~~2mM ATP~~

500mM tris pH 7.6 50µl 1M
100mM $MgCl_2$ 10µl 1M
100mM βME 10µl 1M
30µl H_2O

1 - Add 10x PNK buffer - 5µl
2 - 2.5µl 10mM ATP 1-27
3 - q.s. 49µl 27µl H_2O
4 - Add 10 U/ PNK
✓5 - 37°C, 60'

6 - 65°C, 15'

Ligations
2 sets - one w/o vector - ΔbE
one → E. coli w/ pTZ18 Hinc 2

Sample	Insert	Vector	10x	H_2O	Lig
1	5	0	3	20	2
1+	5	2	3	18	
2	5	0	3	20	
2+	5	2	3	18	
3	5	0	3	20	
3+	5	2	3	18	
4	5	0	3	20	
4+	5	2	3	18	
V	0	2	3	23	

E pCR cloning -J. Stijzki From page 12

9-3-92

Plated on DMSO and lifted

3 #1's mini-prep'd — E2 — E1
2 #4 lifted — E1

all are positive

556
R1 BamH1
1524 2607 1634
2861
2674 1646
H3

Digest reacts with Sal I
to ensure fragments can
be released.

Dates
1-3 #E3 E1
4-11 E1
E1/H3
digests

(961) — (3801)
E3-E1 961 — 3874
E1 2474 — 3874
E2 961 — 2474
365 961 — 3874

Sal I digest

Freeze #1 (E3-E1) E-E3-E1 (B)
2 E E2-E1

does not Sal digest - not frozen

Small portion - 2ul - of mini #1 frozen at -20 for 105 transformation
See "Temporary Storage" file

Map E3-E1 with BamH1, EcoR1, HindIII ~8 Kb 9-7-92
Prepare Sal frag for VV cloning → #5 3' 5'
HindIII — BamH1

Start 100 ml culture for plasmid sequencing
Put into 105 for O — Sunday 9-8-92
See "Temp Storage"

Restriction digest not exactly as predicted except for R1 cut.

Religation of E2 into 191-3Z

	Vector	Insert	10x	H_2O	Lig
VV	2	0	3	23	2
VV+E2	2	5	3	18	2
	4	10	6	36	4

Colonies lifted 9-9-92

See page 21

- E3-E1 Clone digested with SalI
- Add 5ul dH2O
- 2ul Klenow, 20', 37°C
- OH 65°C, 20'
- Remove 10ul as control
- Add 12 u PvuI, 37°C, O/D.

PvuI is predicted to not digest the insert.

Run Control - TEMPStorage Slot 1A with 10ul of PvuI digest

Is fragment still intact?

Does not digest with SacI - page 21

9-24

When digested with SacI and BamHI
- Glycoside portion cut out. - Should have used XbaI
- Humanized fom portion

Redo SacI digest O/D - 10ug #74

→ Do Klenow & ligation O/D.
Labelled 6X-E1 5/B 0-3.
Into JM105, 9-29-92

XbaI added 9:00 20u

Exo III deletion PM. 9-29-92
MBN & AGE 9-29-92
~~Into I~~
Electo, repair & ligate
Into JM105

10-17

E2 Mini's digested w/ EcoRI & BamHI in order to detect insert

Positives in proper orientation will have a ~ 568 bp BamHI/EcoRI fragment

To page 31

Sequence

Sequencing EEE-1 for completion of DE6-265

Presently determined sequence of DE6 extends to EEE-265 at 3920.

EEE-1R extends from 3' terminus up to at 4170. but clone extends to at 2560

∴ Do serial deletions of EEE-1 to obtain remaining 250 nt.

∴ Notes are unclear but it appears that EEE-1 is in Mine 2 site of pT218 - see page 42 of book 3353. or [Bam HI site of pT219]

Therefore do

9-29-92 9/10 digest with ~~Sst I~~ Sst I in React 2

Then digest with Xba I in ~~Bb~~ React 2

Sul run on gel prior to addition of Xba

10-3

Mini prep digested first with Xba 9:45 a.m.

Then 20u Sst I 9/10, Sst 2 accidentally added

Sul on to AGE

30ul aminited and Eco III CORL, 4/ml in 100 µl reaction. 25' samples

After 70°C, spin down with EtOH but no -70 freeze. Pellet for 15'

Additional in predicted Sst site found

Map Site before proceeding

10-10-92

Additional 10ug digested w/ Xba + Sst I

30µl 321 µg/ml E-1

5µl React 2 10x

11µl H_2O

2µl Xba I

2µl Sst I

~~Loaded directly into minigel~~

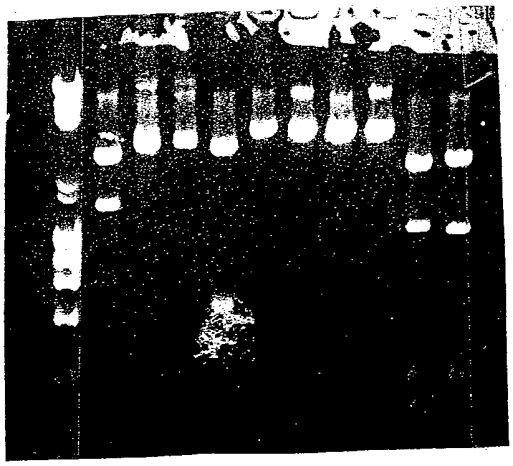
Also digest with Eco RI to eliminate E-1 as long as pT219 RI Site

Is in pT219 Bam Site.

?

Would explain Sst I Xba result

lapping E-1



Bam H1
Sst1
Pst1
Kpn
Xba1
EcoR1
Ava1
Sma1
R1/H3
R1/Sph
Bam H1/H3

λ ΦX
Bam H1
Ava1
Sma1
Pst1
Kpn1
Sst1
EcoR1
Xba1
R1/H3
X/H3

∴ must be in Bam H1 site
Compare X/H3, R1/H3, Bam H1
also supported by confusion on page 50 of book 3353.

∴ Cut out Bam Fragment
Blunt end into pT218 HincII
Use Kpn1 or Sst1 for generation of deletions

Tube 47 213ng/μl
40ug DNA
5μl 10x High
2μl H_2O
3μl Bam H1

37°C, 1hr
Blunt end
Purify & ligate

10-17-92
Into pT218.
Digest with H3
Desired plasmid will yield ~120 + 300bp fragment
i.e. with 3' end near primer binding site.
If using other orientation HincII should delete most all of unknown sequence.

MM26 sequencing

9-28-92

MM26 digested with ~~Sst2~~ Xba I - 30ug.

9-29

5ul to agarose gel to check on completion of cutting.

40ul adjusted to 100ul Exo III reaction

8ul samples taken out 20" intervals and DH 70°C, 10.

EtOH precipitated : Dried.

- Suspend in ~~30~~ 15ul H_2O
- Add 5ul MBN cocktail

2ul 10x MBN
5u MBN
q.s 5ul

MBN = 75 U/ul

15 portions 30ul 10x
44ul H_2O
1ul MBN.

37°C, 20'

1% AGE 1.5ma, 100V.

Elute
Repair
Ligate
3:30 1U ligase / 30ul reaction

Into DH5α p.m. 9-29-92

9-30

2 colonies each picked from 0-8, 11. 9+10 gave no colonies.

0-1 All - A samples sequenced and run on BRL 6% gels.

0-3 Into JM105's

Deletion of MM39.

5' protected end 5sI2 digestion
Submerse gel, Additional 5sI2 added 9:00 10-2

2nd digestion (-BAM HI)
Repeated 10-6-92 - Small amount of uncut circle remaining after 5sI2 digestion
MM 23 (Apa I)
Acc I & Cla I) Added 12:30 10-6-92

10-4-92
23 - from transformation complete
Induction - 9hr

After MBN treatment
- ~½ of sample is in orange!
- ½ 0-gel: EtOH
Repaired
Ligated without Age

→ Samples look very clean but extent of deletion is less than expected
∴ Repeat & use longer serial time points. - First set transformed & discarded 10-7-92
Start digest 10-5-92
Apa I in React 4
10ug in 50ul reaction
12ul DNA
- Small amount of undigested left
10-6-92 Additional Apa I added 11:30
Colonies picked

10-6-92
After MBN digestion, half of #1 & #10 remain on mini gel, freeze remainder
If DNA is clean and single band is present, EtOH & repair/ligate/transform.
Sample 10 ~ 2KB
∴ Repair 1-8.

EEE, WEE cDNA Synthesis

Using BM reagents for first strand synthesis

Buffer 1 is 5x

20µl buffer 1
4.5µl RNAsin - BRL
10µl dNTP - M.P. 5mM
10µl random primer
5µl ^{35}-dATP
20µl RNA - 10ug EEE
28µl H_2O
4µl RT

60', 42°C.
2µl samples (P20)

		cpm net	%	nM inc	ng cDNA
8µl 500mM EDTA	+/-	900,000			
92µl H_2O	+/+	6K			
Phenol 2x	+/+	6K			
133µl 5M NH_4OAc	+60+				
800µl EtOH					

No incorporation

-70°C, 30'
-Wash and pellet.
-Repeat.

10-12-92
Standardization of RT reaction
Why no incorporation in several attempts?

Using Ladder RNA - 2ug
Do a series of RT reactions to maximize cDNA synthesis, use random primer + oligo dT

Standard Buffer cocktail

50mM	tris pH 8.3	5µl 1M
10mM	KCl	7µl 1M
10mM	$MgCl_2$	10µl 100mM
10mM	DTT	10µl 500mM

✓ 5/~~5~~ ul RNA ← 1 ng/ul

Primer @ Higo-dT 3ul @ RP-5 ✓ BM from kits

✓ dNTP 10ul

✓ RNAsin ~~2.5~~ 5ul - BRL

✓ Buffer cocktail ~~24ul~~ 32

✓ H_2O ~~36 38 46~~ 5ul 34/~~36~~ 49 46ul

✓ RT 4ul

✓ 35S 5ul

5ul samples 5 min RM T, 2 hrs 42°C.

12:00

Sample	cpm	net	%	nMole Inc	ug cDNA
1A	500171				
1B	1096				
1C	9505	8409	0.0168	336	1.1096
2A	417617				
2B	1517				
2C	10757	9240	0.022	444	1.45

Approximately 20-25% conversion

Reasonable

What RT

to page 3

Subcloning 6K-E1 from pJ2 Tube 74 10-4-92
6K-E1 = pJ218 does not cut out with SacI
Because plasmid homologous with poly
of oligonucleotide that 3' SalI site is not there
∴ Remove insert with SalI + XbaI digestion
Digest with PvuII

All three done together repeated 10-5
~7.5ug

When complete, fill in cards justifying fragments

10-5-92 ~~SacI~~ dUTP
2ul Klenow - 37°C, 15', ~~0~~ - Purify fragment
Religation of E2 into 191-32
Not known if fragment is blunt ended - notes don't say
∴ Blunt end before doing ligation Tube 61
~~12ul H2O~~ 5ul fragment
2ul 10x Salt Buffer
1ul 5mM dUTP
4ul H2O
1ul Klenow
37°C, 20' incubate
70°C, 15'
Ligation as before (page 13)
Into JM105 10-5-92

Ligation as on p.g. 13 10-5-92
New batch prepared.

10-10-92
Ethanol precipitated after digestion

Suspend in 18ul H2O — 3ul to minigel
To remaining 15ul
2ul 10x buffer
1ul dUTP 5mM
1ul H2O
2ul Klenow
37°C 20' 2:50
Loaded directly onto minigel.

AGAIN - MATERIAL BADLY SMEARED - DISCARDED.

10-11-92
Repeat triple digest

Phenol extract
EtOH ppt.

T4 repair

✓ 5ul 10x T4
✓ 4ul 5mM dNTP
✓ 40ul H_2O
✓ 1ul T4 Pol

After adding components - dNTP
✓ Incubate 37°C, 1 minute
Add dNTP
✓ 37°C, 20'

DH-70°C 15'
Ortogel
Single band eluted and inserted into
P191-32 as on page 13.

~~Redo increasing Pvu I digest.~~
10-14 Redo Pvu I digest of blunt fragment
Tube 62

Ligation background much to high!
Initial Pvu I ~~digest~~ digestion left
largest band too dark.
└ pT2 still present?

10-17-92 Repeated Cleanup and
Ligation

10-9-92.

Plasmids from mini's of MM23 Exo's OH⁻ denatured and under EtOH at -70°C.

Second set of MM39 ~~-20~~" samples taken starting 2 minutes after addition of Exo III — 10-10-92 5μl Exo III
Stopped at -70°C, 10' and EtOH added.
Stored at -70°C until returned from Bet!

Terminated.

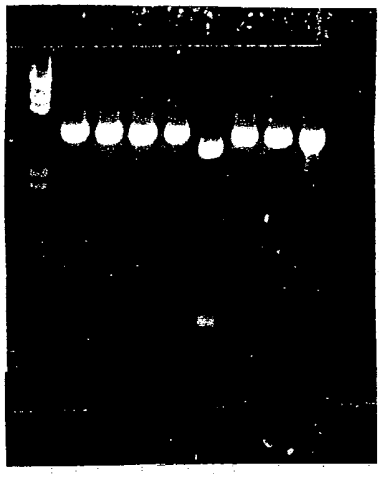
10-10-92

Restriction maps of MM39

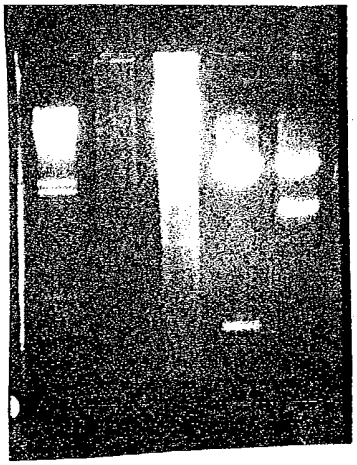
Redo Hind3, Bam digest to re determine Size of insert.

(23 ~ 0.3Kb)
39 ~ 1.6 Kb.

λ
Kpn I
Hind 3
Xba I
Sal I
Hinc 2
Cla I
Apa I
Acc I



λ H3
x
x
23 H3/Bam H1
39



10-11-92

Work on MM23 discontinued

It appears that problem with deletions in MM39 due to Exo III. Sufficient activity seen only when [Exo III] is doubled.

∴ 10ug digested with SstI + Bam O/N in 50μl BRL 2

37°C, 3'

10µl - 65u/µl added.

65µl samples taken at 20 sec intervals
DH - 70°C, 11.5 min
0.5ml EtOH
Pelleted

Digested w/ 5u MBN/sample
20', 37°C
DCE resolved.

Fractions 1-12 Eluted
Prepare ligate

10-14-92

SST2 is not a resistant 3' end
∴ find new enzyme for generating termini

Xba filling w/ 5-dNTPs.
Followed by second digestion with BamHI

Digest 20ng in 50µl reaction
25µl DDA
5µl 10x Hi-fi
15µl H2O
5µl Xba

Remove 2µl aliquots to 20µl and add Hi-fi

+ 4µl dNTP (2.5mM) w/ 5-dG, 5-dC
+ 24u Klenow
20', 37°C.
70°C, 15'

Remove 2ng to serve as control on Exo III treatment. Is it resistant?,
T4 m.d. Br -

from p.g. 24

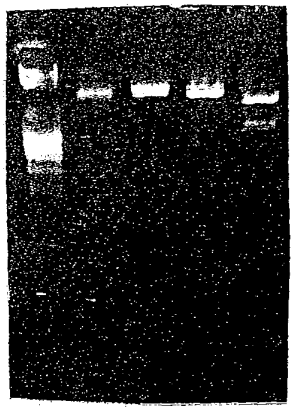
Mapping MM39

** does not cut vector

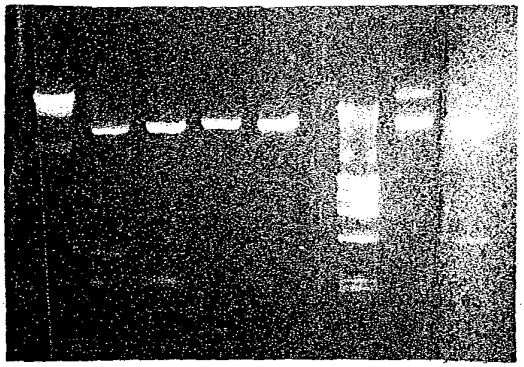
λ
ΦX

SstI
BamHI
*Bgl2
Eco2

Smaller frag
~ 600bp

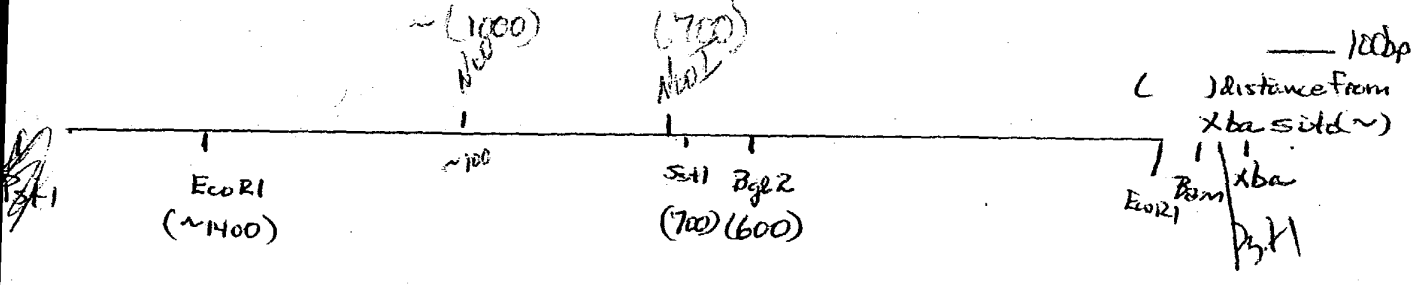


λ
*Sty I
*Nco I
Pst I
Xba I
λΦX
*Nhe I
Xba/Bgl2



Unmapped site *Sty I,
*Nco I,
*Nhe

λ-BstEII
MM39
Nco I
Nco I / BamH
Nco I / HindIII



10-14-92

MM 39 Sequencing Deletions from pg. 24

✓ After heat inactivation of Klenow

✓ Remove 5µl for Exo III control - label XbaI/B C
(2µg in each of these)

✓ 15µl + 3.5µl 10x High + 30µl H_2O + 1.5µl BamHI (39 X/B/B)

✓ 15µl + 3.5µl 10x High + 30µl H_2O + 1.5µl Bgl2 (39/X/B/Bgl2)

Digest O/N.

10-15

Bam Digests done first labeled 1-12 Bgl2 digests label A-J

✓ 50µl restriction digests
✓ 10µl 10x Exo III
✓ 30µl H_2O
✓ 37°C, 5'
✓ 10µl Exo III

Include Klenow fill in control
✓ 5µl DDA
✓ 3µl 10x Exo
✓ 19µl H_2O
✓ 3µl Exo III

10µl sample - time 0
w/ 10th point from Bam digested material

70°C, 10'
500µl EtOH
Pellet
Dry

Mung Bean treatment
Standard Protocol

Add -
Running paired controls

λ C C1 x 1/10
Vector is resistant,
No deletions evident
Do O/N digestion w/ second enzyme

repair period and second site cutting was not OK.

- Start second Xba I digestion
- Repeat for 5 minutes instead of 20'
  Phenol extract

- Phenol extraction prior to each enzyme
  failed to yield desired deleted intervals

Is one of the buffers bad.
Redo 1432 treatment w/ Bam & Hdc
10-16-92

Exo III

Sug's fragments.

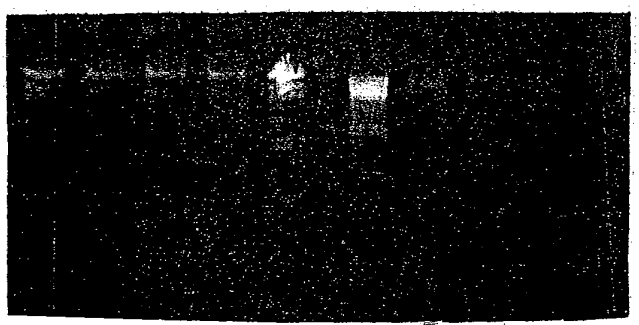
- Set up 100 µl Exo III Reactions - 3' time points
- After heat treatment
- Run fragments out on gel.
  Smallest fragments should disappear first.

5µl fragments
5µl 10x Exo III
25µl H_2O
5µl BRL Exo III

5µl samples at 3' intervals

Treat 10x Exo III
500mM tris pH 8.0
100mM ~~BME~~ DTT
50mM $MgCl_2$

Exo III Test
1-4 BRL
7-10 Stratagene
in BRL
Buffer



from page 28

Exonuclease buffers had no effect
Exo 3 - both batches worked fine!
Repair w/ buds 5-2x PP, g, c. 1µl/20µl Rxn, 10'
∴ Additional controls
After Xba I digestion
① Remove 5ng for repair, heat inactivate, Exo 3 ← O/N (-20°C)
② Remove 5ng for control. ~~Exo 3~~ ← -20°C
③ ~~Cut 5ng~~
Remove 5ng for repair
Heat inactivate Klenow ✓ (O/N (-20°)
Exonuclease treat
④ Remove 5ng for repair
Heat inactivate Klenow → Didn't Bgl 2 cut?
Bgl 2 cut - O/N 10U ←
Exonuclease treat
⑤ Remove 5ng
Bgl 2 cut - O/N 10 Units ← ✓
Exonuclease treat

Increase Exo III treatment to 1000 Units - 10µl in 100µl Rxn, using BRL Exonuclease Buffer

Xba I digestion
50ng in 200µl

Samples 1-5 20µl samples, remainder frozen in temp storage at -20

9-17-92
Run 2µl of O/N Bgl 2 digests to insure cutting occurred.
Complete

Remove 2 ng - 5µl of each
Add 10µl H_2O
5µl Exo Cocktail

2µl 10x Exo III - BRL
2µl Exo III - Stratagene
1µl H_2O

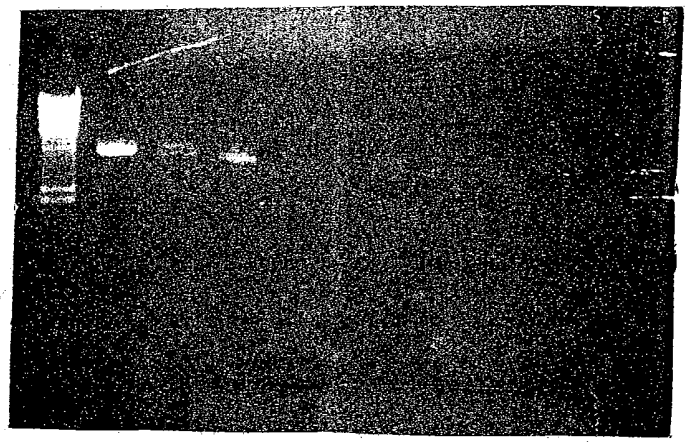
37°C, 3 minutes
- Heat inactivate
- Exo III spot

Samples suspended in 15ul H_2O
+ 2ul 10x ~~Exo~~ MBN
10 Units of MBN
@ 5. 20ul.

Run on 2 minigels

1 - 1+ 2 - 2 3 - 3 4 - 5
- denotes beginning of Exo III treatment

① No digestion as predicted
② 2+ not visible
④ ~~wasn't~~ B.f.2 did not cut
⑤ 5+ not visible



1 - 1+ 2 2+ 3 - 3+ 4 - 4+ 5 5+

It appears that the second cut doesn't take place if 5-dATP are present

Run the remaining undigested 4 & 5 samples to see if B.f.2 did cut

B.f.2 did digest

Is exonuclease buffer bad?
Or does not function in presence of 5-dATP

~~MBN testing:~~
~~Repeat Exo III treatment of 1 & 4~~
over a time course
~~Treat half w/MBN, half w/o~~

1 Water 7ul
1 Insert 5ul
1 10x Buffer 2ul

J. Stuyki

E2 into pTZ19U-3Z

Digestion of 12 minis with Bam / EcoRI

Colony 3 = positive orientation
10 = negative orientation

from 19U-1

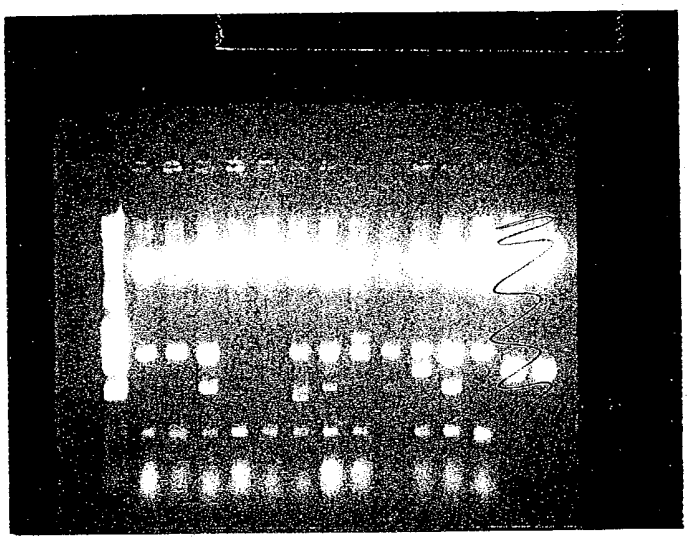
→ ~600 bp R1/113 Frag

→ ~900 bp R1/113 Frag

Frozen slot 219
220

Colony 1 chosen for stock master preparation
Checked for Sma I & Bgm HI digestion.
Maxi's of 219 & 220
Start 10-17-pm.

Digest 2's 10 with Bam/RI



Both DOA's & PAT 10-20-92

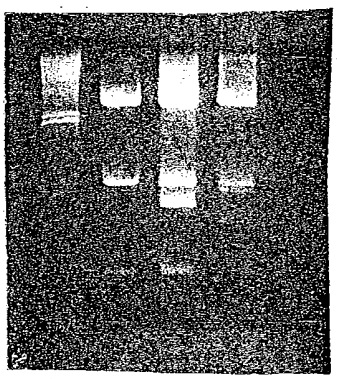
10-19-92
6K-E1 in 191-3Z → DH5α
10-20 Lifted & pretty bridged 9x12".
Probe synthesis.

Initial probe synthesis not successful.

10-21-92
Probe synthesis
using 7μl from Blunt 6K-E1 fragment
Tube G2

(7μl DOA ✓
7μl H2O ✓
DH
7μl dATP-DA
7μl reaction mix
7μl BS-dATP
7μl Klenow)

10-21-92
191-3Z E2- / E2+ Bam/EcoRI



Run column 2150 (and 2)

10-22

Blotted and probed PHO

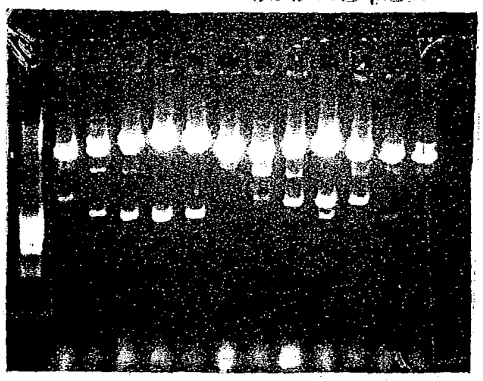
10-23 Washed and readied-to-go-again

10-24 Minipreps probed & Minis prep'd
digest with Bam HI
Sequence products dried at ~260

Positive orientation will yield ~1700 bp frag
Negative ~ 1300 bp with respect to promoter

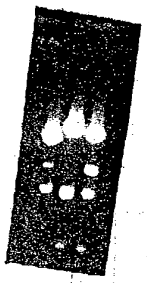
Several clones of each identified
#1 = bKE1-191 (+) Slot 225 — Suspect !!! See below
#4 = bK-E1-191 (−) Slot 226
#9 is (+) — 225 is known Contract

Backbone on #1 looks too small
Check by digestion with
~~Bam HI + Eco RI together~~
Eco RI alone.
Do 1, 4, 9.
9 as an alternate
to #1



RI digestion should
release entire
insert cut in 2 pieces
along with flanking
pieces
HindIII/Bam HI will orient
insert and identify vector
due to 1000 bp vector fragment

~~It~~ appears that #1 is not the
proper construct ~~re the~~
#9 is correct
∴ #9 saved as bKE1-191 (+)
+ orientation
# is a negative, non recombinant



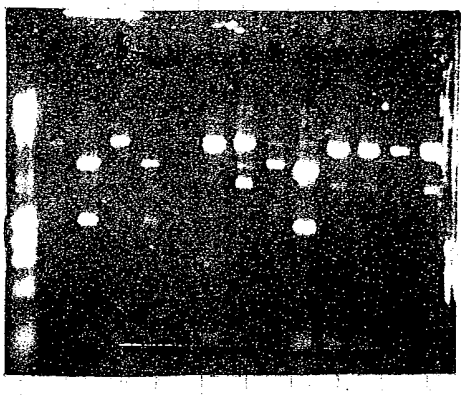
#9 is confirmed

EE-1 subcloning from page 16

3 colonies picked and digested with Hind3. in pTZ18-Hinc2

#3 is orientation 5-3'
#2 3'←5

Frage #3 in clot 222 EEE-1 —
#2 " Slot 223 EEE-1 +



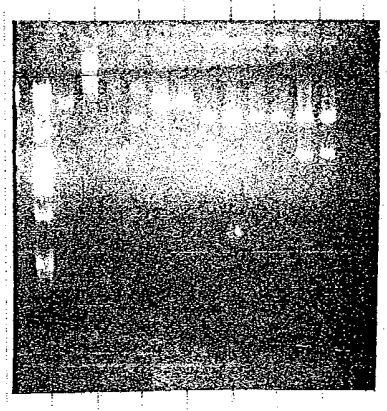
Digest #2 with Hinc 2 to
ucing will delete most 3'
E-1 for easy access to predicted
ap in known sequence.
Requires Hinc 2 - Sma I digest to provide
ompatible ends.
— Done
79-92 Ligated and with JM105
0-21-92
6 colonies picked for mini preps
Digest 1-6 w/ Sma
1-6 w/ Eco RI / Hind 3

Looking for clones w/o Sma site
and likely larger internal band.

Picked #2, 3, 4 for sequencing

#4
1-6 Sma I
1-6 RI/H3

Run on BRL 6% gel.



Sequenced and provided
eeded sequence to complete pEE1-5

#2 Frage as E-1142, Slot 324

Exo III of MM39 by the kit

- 40ug MM39 digested with XbaI in 250µl reaction - Checked by Ake 1:30 p.m.
- ~~Add additional 250 µl High Salt buffer when complete~~
Heat 75°C, 15'
Add 2µl 1mM thio-dNTP
+ 6u Klenow polymerase - BRL
Room T - 10'
Phenol extract
Ethanol precipitate, Suspend in 100µl (~4ug/µl)
Check filling by 15 min digestion with 20u Exo III using 1ug DNA. Look for shift

Do second digestion in small volume - O/N - Bfr 2
Bfr 2

10µl / 100 saved for Exo3 control

5µl of Bfr 2 digest → 46E to monitor completion of digestion

After MBD + EtOH treatment.
No detectible DNA
- Terminated -

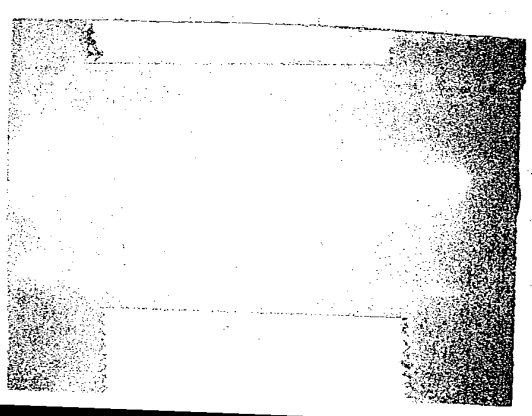
10-19-92

Clone 30ug with XbaI - Sample 1 2ug
Repaired with Strategene 5dCTP Klenow
- 2ug Sample 2

EtOH extract
Bfr 2 digest O/N. ~ 20ug in 30µl digestion

Delete w/ large amt - 10µl Exo III and results - 20second time pts

It appears that significant amount of Exo3 two population of molecules are present - Seems unlikely
Rate of Exonuclease digestion is very slow also



When repeated with same DNA

Smaller band reaction with 2 enzymes one plasmid deletes. Wolff

Is this 3?

Repeat large scale digestion using Bam HI site as second site!

Also do Dig w/ BglII only

10-21-91

→ Frozen O/N at -20° before Known Repair

1 ug of Xba also digested w/ H3. Does this plasmid look as before?

See page 23

Plasmid deletion of MM39 10-21-92

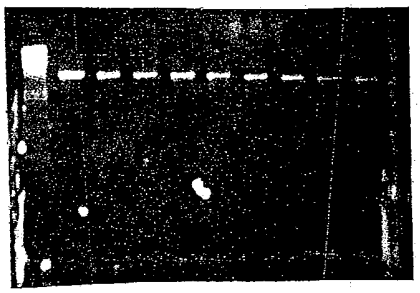
Apa I ~~Sph I~~, Hind III deletions

Digest 20µg MM39 in Low Salt with ~~Sph I~~ Apa I - 30µl

Add 3µl 1M NaCl
40µl Hind III

Do standard Exonuclease treatment with
Ut5B exo buffer & Stratagene Enzyme
20 sec time points

Voila!



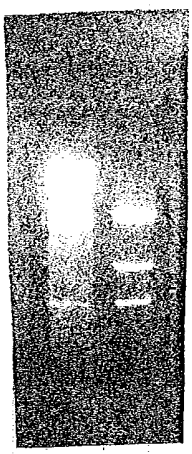
9 fragments harvested.
Kinetics still look a little slow. ~~Repeat~~ Probably appears that way because gel was run only a short time.

Into MM105's 11.10-25-92

See accompanying photo on page 38.

Because Xba Bam deletion still only represent half a plasmid being digested.
Digest ~ 1/2 of Bgl 2 cut material w/ HB to see if 2° digestion is going to completion.

That is exactly what's happening. → ~~not happening~~.
After addition of Hind III, a second fragment corresponding to the remainder of the MM39 insert is released. If Bgl 2 was cutting only half the DNA, a third fragment the size of the intact insert should have been released also.

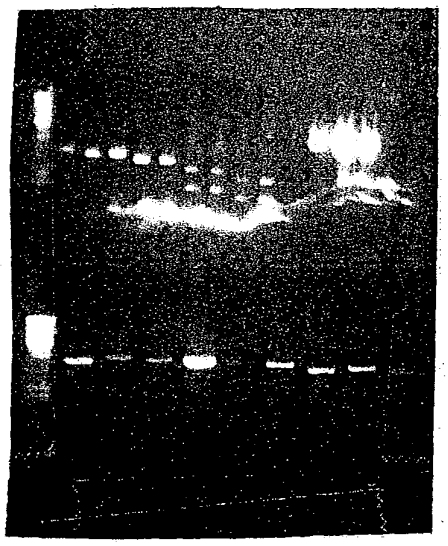


It may be worthwhile to try a second site. Or - Is it possible the 5-dNTP inhibit the Exonuclease.
Do it over! 5 min Columns

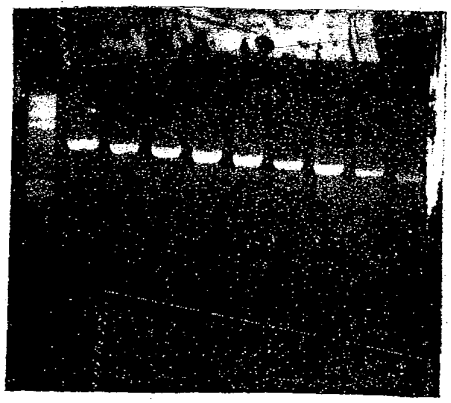
Isolations picked from time points 1-9
Mini's prepared by Alk
EtOH ppt'd.
Resuspended in 2 ml H_2O
① Resuspension of deletions
Digest 3 Rxns of each with B from #1.
Digest 30', Inact 0/N each
② Do alkaline denaturation.
Gel DOA
2 ml 2M NaOH
10'
Add 2 ml 2.5M NaOAc
1 ml H_2O
75 ml EtOH.

-70° 25'
Pellet'd Day.

When Base Hydrolyzed
#1-9 equivocal. 10-18 are definitely showing evidence of deletions. Doublets appearing in 15-18 suggest that deletions had extended into down stream portion of polylinker



Second set of plasmid deletions processed 10-28-92



MM39 cont'd

From page 38

Because Sequencing is now going so slowly,
Subclone the BamHI/H3/Blunt frag of MM39 into pTZ19 Sma.

Digest mini's with BamHI, this will indicate which direction the insert is in.

CCA 1B ——— BH 666 → regenerates Bam Site.
Use opposite orientation so that BamHI can also be used to generate susceptible end.

Restrict — ~~PstI~~
Susceptible — ~~Xba~~, BamHI
Sac ?

10-30

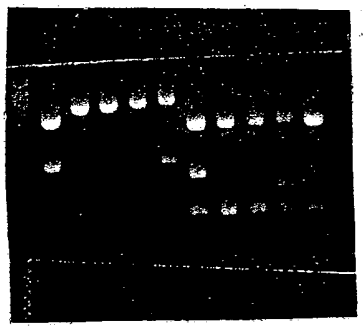
- 7ug MM39 digested with BamHI + HindIII — Qiaexpress
- 2 ul 5mM dNTP mixed
- 1 ul (6U) Klenow
- RmT, 10', then dirty to gel, 2 lanes.
- ~~DNA~~
→ AGE.
(No need)

11-2

The initial set of detections sequenced lack the 1st 700 nt.
— A second set were prepared and the 5 mini preps were prepared.
In order to see if these 700 nt were present in the earliest time points, they were digested with 5ul of each
① EcoRI
② ~~SmaI~~, ~~PstI~~ Pou2 brackets cloning site based upon the presumption that the SK+ vector has the same fidelity orientation as KS+

11-39V

λ BstEII
MM39 - EcoRI
- 2B
- 2A
- 1B
- 1A
As Above Rxn 2



The Results with 1A don't give!

MM39 HindIII/ NcoI Fragment purified
Probe preparation
✓ 10μL DNA
✓ Boil - 10', Quench ✓
✓ 2μL reaction mix
✓ 2μL dATP-dCTP
5μL α-pdGTP
1μL Klenow

Also for cloning
✓ 10μL DNA
✓ 5μL 10x React I
15μL dATP
✓ 1μL Sme-H_2O
✓ 1μL Klenow - BM - 2U
Rxn T 10'
ΔH 65°C, 10'
Ligate 5μL into pTZ 18 Hind 2

✓ Insert 5μL
✓ Vector 2
10x 3
✓ H_2O 19 Lab JM105
✓ Ligase 2

6 white colonies
of ~ 30 total
all picked

Assembly of PEB-265.

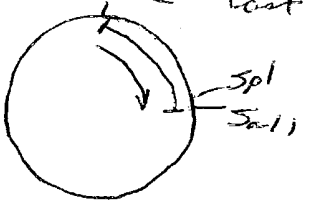
PCR clone E265-Sal extends to nt ~ 2452
EEE-E extends upstream ~ 2200. 2520

With in the overlap is a unique Sp11 site which can be used to join the two.

Two approaches Xba1 + Sal1 are not contained with in PEB-265 sequence.

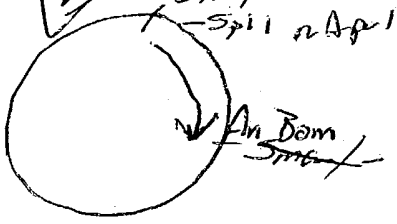
A) 3 Point ligation

E265-Sal 2.7 Kb Sal fragment in Sal site of pTZ18
nucleotide #1 = 166 of PEB-265 sequence
last nt of insert = 2184 "2520
Harvest ~2.7 Kb Sal1 – Sp1 fragment
Use Pst1 to digest vector down #38



pTZ18.

EEE-E 2.0 Kb fragment in Bam site of pTZ19 #47



pTZ19.

HARVEST 2.0 Kb Sp11 – Xba1 frag
Dug Xba1 digested 11-9-92

Three point ligation into Sal1 – Xba1 pTZ19. Will allow use of T7 for plus sense RNA.

B) Vector reconstruction

→ Prepare 3.6 Kb frag Sca1 → Sp1 1 of E265-Sal in pTZ18 #38
+ 3.5 Kb frag Sp11 → Sca1 of EEE-E #47
(1 ug of each, 55C.)

Combine & ligate.

10-30-92

Digest 1ug of each plasmid with Sp11. Old enzyme may not work.

See page 97

E265-Sal digests cleanly
E-E doesn't seem as clean as usual.

10-31

Digest 5µl E26-Sal with Sp11 in High Salt Buffer.

DNA tube 38.

Also do control Sal I digest to check ID of plasmid.

Sp1 seems to be only partially cutting ~60%

∴ Add 1µl additional Sp11
1µl Sal I

To ensure that Sp1 did in fact cut, digest 1µg of Sp1 digested material with Bst1. (3µl) Bst1 should not cut within this portion of PE6-26S but should liberate a ~2.5Kb fragment only slightly smaller than seen from Sal I digest previously.

It seems that Sp1 is cutting only a portion of the plasmid yielding two closely migrating fragments.

This suggests that the Sp11 – Sca I approach may be the better of the two.

11-1-92

Release what appears to be clean Sal-Sp1 fragment
Freeze at -20°C

EEE-E Xba
E26S-Sal
Sp1 I/Sal I/Pst I

Start O/N
Sca I – Sp11 digest of E26S-Sal
5 µg – Tube 38

Also EEE-E Tube 37

Terminated here

To page 97

Eastern cDDA cloning repeat from pg 20

Why? To extend the cDDA map into the NS region using random primers and blunt end cloning.

PE6 RDA as substrate

? 2 preps by CsTFA extraction - did it work?

Agarose gel.

Mercury gels.

1) Start with cellular RDA

2) What ing to use - horizontal with a pump?

Electrode buffer -

from Maniatis -

11-15-92

EEE cDDA

RDA - from CsTFA

20µl RDA in H_2O	3-20	
42°C - 2'	3-20	
5µl Random primer - ~~5µl~~	3-20	
10µl 5 mM dATP	3-20	
5µl RDA sin - BRL	3-320	Primers
32µl Buffer cocktail	3-20	5µl 1M tris pH 8.3 DMD
		2µl 1M KCl
21 µl H_2O	24-3-20	1µl 100mM $MgCl_2$
4 µl RT 15 Amv RT 007		10µl 100mM DTT
3µl ^{32}P-dCTP		

5'-Rm Tx 55' 42°C - 1½ total channel 2

2µl samples	cpm	net	%	nM	ug cDNA
0 -	767481				
0 +	312				
120 +	19064	18752	0.024	4.89	1.6 ug ~15%

WEE	
~~+~~	+
+	+
+	+
+	1
+	2
+	✓
+	24
+	

5µl EDTA

8µl sample → gel

	Channel 1 cpm = ^{32}P =
8011 2x + 100µl TE	50143
CHCl3 1x	171
Add 133 µl 5M NH4OAc	11367
1 mL EtOH	

11769 ✓

2nd Strand Synthesis

Protocol from Book 3353 page 44

2nd strand buffer

20mM tris pH 7.4	~~1M~~ 10µl 100mM	=10	✓
5mM $MgCl_2$	2.5µl 100mM		✓
10mM $(NH_4)_2SO_4$	5µl 100mM		✓
100mM KCl	5µl 1M	per 50µl reaction	✓
250µM dNTP	2.5µl .5mM		

10 reactions made and stored at -20°C

e 19.~~5~~ 27.5µl substrate in H_2O	22	
~~25~~ 16µl cocktail	25	
e. ~~3~~µl ^{3}H-dCTP	–	
µl 1µl RNAse H	1	
.0µl 2.0µl Pol I = 20 Units - Bel	2	

1µl samples. channel 3 = ^{3}H 0-700

	1:15	0-	3632	
10:30	0:45 2hrs 2.5hrs 12°C bath	0+	3866	
11:40	1hr 22°C bath	2hr	8923	5057 0.139 ~ 2.2ng

5µl EDTA

148µl H_2O

~~DOH extract~~

~~- DOH extract~~

~~- Spun column~~

Abs in channel 2 in = ^{32}P 8916 cpm remaining

% of beginning?

20µl NH_4OAc

500µl EtOH

Pellet 3 Dry ✓ to page 45

Vector preparation

- pT218-Hnc2 digested
  10ng in 100µl, 15u
- 5µl 1M tris pH 8.3
- 2µl CIAP 20', 37° (28u)
- DOH
- EtOH
- T4 Repair

11-16-92

Vector Repair — from page 44

41.5 µl H_2O
5 µl 10x T4 Pol
2.5 µl 5mM dNTP
2 µl T4 Pol — 10 µl
37°C, 20'
5 µl EDTA
45 µl TE, 5 prm Columns. — 145 µg/ml — OD_{260} = 1.505 / 280) a little low?

T4 Repair of cDNA
As above, 1 µl T4 Pol, 20', 37°C.
2 µl EDTA
DH ~~70°C~~ 5'

→ Resolve on agarose : electroelute.

45 µl TE
~~Ø~~ Ø
$CHCl_3$
Spin column
132 µl came through

1.3 µl counted — Channel 1 = ^{32}P = ~~0-700~~ 700-1000 = 3085 cpm
≈ 1%
3 = ^3H = 0-700 = 4937 cpm

% ~~note~~ cDNA still present

Initial cpm ~ 11367 cpm / 2 µl = 2% of total
Final = 6170 / 2.6 = 2% " = ^{32}P 5+% or remaining ≈ 864 ng

Final 6176 ~~7878~~ > numbers don't jive.
Initial 5057 see note below & could this be due to additional incorporation during T4 repair?

13 µl NH_4OAc added
+ EtOH to cDNA — -20°C 9h.

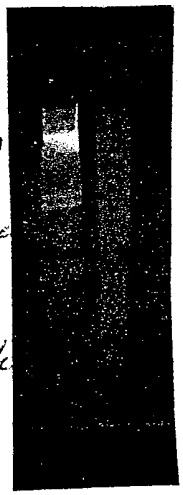
When samples from 1st strand were recounted
^{32}P in first strand
0-1000 window of final timepoint = 18133
0-700 channel 3 = 6836
700-1000 " 1 = 11367
" in subsequent counts
60% of cpm in channel 1 are found in channel 3.

Therefore in final cpm of ds cDNA
① Assume that all of Tomwood window are ^{32}P = 3085
Then 1.60 × 3085 = 1851 of cpm in channel 3 or
^{32}P,
then final ^{3}H = 4439 − 1851 = 3088 cpm ^{3}H
" 1% of 12

6176 = 2%

11-17-92
EtOH precipitate pelleted.
~~Ligations~~.

Suspend in 30µl TE
Fractionate 20µl on Agarose
electroelute.
Broad ~~pa~~smear of DNA
fractionated into 8 fr
1 – Largest ⟶ 8.
EtOH ppt'd.



1 BE gel off first strand

Ligation of inserts
Vector preparation pBR328 Hind 2 #30

Suspend cDNA in 20µl sterile H_2O

Sample	Vector	Ins	10×	H_2O	L
Vector	2	0	3	23	2
1	2	5			
2	2	5			
3	2	5			
4	2	5			
5	2	5			
6	2	5			
7	2	5			
8	2	5		18	
Rec B-Sma	2	Vector 3		18	
		non fractionated			

1µl = 300 cpm / clone

~5-10% chosen

1µl = bkgd cpm

~50% white

2.1 DNA

4 more 53

11-17-92

Mayaro Preparation - J. Thomas

8 T150 flasks
at 3 days after seeding

Stock virus MRC-5 grow Mayaro
19-April-89
FJM

-1ml diluted to 10 w/ EMEM + 5% FCS
- 1ml to each flask
- 4 hours ~1hr
- 20ml media added
- 37°C, 5% CO_2

Remainder of virus stock frozen in slot 231

VIRUS HARVESTED AM. 11-19-92

1ml frozen as reference in slot 232

- Virus pelleted, 2hrs 27K SW28
Suspend each tube in Single TNE with gentle stirring

Sediment of 20-55% Sucrose in TNE, 4hrs, 28K, 4°C
O/N

- Very thin band collected and pelleted

Suspended in 200µl TNE → 20µl for PAGE 13% mini P-gel
+ 200µl TNE
+ 20µl 10% SDS
~~Ether~~ x3
$CHCl_3$ x1

200V, I = 30mA
~3.25
~1hr

0.1 Vol 2.5M NaOAc
+ EtOH → -20°C,

2nd preparation 11-28-92
Held in freezer 0/0.

3-6-93

Repeat virus preparation

10.10 flasks MRC-5 inoculated with 1 ml 10^{-1} dilution of MRC-5 prepared Mayaro virus of 19-April-1989 – ETH – 1:45 p.m.

Rolled 20'

Fed 30 ml MEM + 2.5% FBS + glutamine + GPS.

- 2 drop P.I. – little CPE.
- HARVESTED AND PURIFIED (PEG : NaCl)
- DOUBLE BAND HARVESTED AND PELLETED
- RESUSPEND IN TNE
- 10 µl RUN ON 10% gel – minigel

MW VEE MAYARO

Started 100V, 31mA t0 : 10:35

raised to 200 V, 58mA t0 = 10:50

3B had run off (by 11:30

0/N CsTFA Run

1:20 dil of 400 µl pellet

OD_{260} = 0.06 = 2.4 ug/ml

x 20 = 48 ug/ml

3-1-92 x 0.4 ml = 19.2 ug RDA

Frozen in TE Buffer – Slot 254

Additional 10.10 flasks of VERO's INFECTED WITH 1:20 dilution of MRC-5 grown Mayaro virus.

3-13 Harvested

- PEG'ed
- 4 hrs, 15-55% sucrose

Very clear, only virus was visible in gradients

RDA stored under ethanol

total yield 19.2 ug in slot 255

11-17-92

Chik Recot cDDA Synthesis
PCR extracted from material left by
Ken Sanroe
Pelleted and OD260 determined.

to page 71

11-18-92

Hg Agarose Gels

10x Buffer = 500mM Boric Acid
50 mM Na Borate
100mM Na_2SO_4
pH = 8.15

1.2% Horizontal mini gel
V_o = 100
i_o = 105 i_f = 130

t_o = 1:05 t_f = 1:55
Looks as if 1 hour will cover the length of gel. Gel gets warm at these settings!

100ul SF9
100ul EG RDA's pelleted & dried.
Suspended in 15ul Hg⁺² sample buffer

~~H50 sucrose~~
Ladder - Vero - SF9. ~~SF~~

SF9 not visible
Vero 28S 18S are visible but very streaky.
Ladder is a contamination smear.

Redo after autoclaving agarose & buffers.

1 = λ DOA
2 = BRL Ladder
3 = BRL Ladder
4 = Kb ladder — DOA or RDA? BRL
5 = BM RDA Mol Markers — 3ul

80V, 100mA
Same buffer as 1st run.

Try vertical format with large bottom reservoir
1.2%, V_o = 80 ✓
i_o = 46mA
t_o = 3:30 t_f = 4:35

Perfect
Some edge effects

from pg 46

pE6 cloning continued

cDOA probe synthesis

10µl RDA preparation ✓	40C / min	x	x 56°C, 2'	x
2µl Random primers ✓		x	x	x
10µl buffer cocktail ✓		x	x	x
5µl dATP-dA ✓		x	x	x
2µl RDAsin		x	x	x
5µl ^{35}S-dATP ✓		x	x	x
8µl H_2O ✓		x	x	x
2µl RT ✓				x
5, 8mT, 42°C, 1hr.				x

5µl column effluent = 57Kcpm. Prepare 100 additional with more RDA. Try 20µl — is response linear?

50µl RDA → 257K cpm /gel

Boil and hybridize 60°C, Homemade buffer protocol.

Probe added Friday 12:00 noon.

When starting material was analyzed on agarose gel, 1kb gave intense smear-like Agarose gel. RDase treatment resulted in total solubilization. ∴ Prep is cleaning DOA but is fragmented.

11-22-92

Numerous positive colonies

Many more in unfractionated material digested to pre 13.

Some scattered's in fractionated material — will pick these first. ←

17,8 · 18 total

11-23-92 R1/H1-83 digest.

Filter blots returned

Remainder of fractionated material ligated to next preparation of blunt ended Hpa 2 cut - TTR plasmid pT 218. #31 7 ng/μl
fraction 1-8 only
+ 2μl of non fractionated material ligated to 4μl vector - denoted pTR.

Eluted material in 15μl H_2O
Add 3μl 10x
4μl vector
6μl H_2O
2μl ligase & tack.

Vector control
4μl Vector
3μl 10x
21μl H_2O
2μl vector

pTR
4μl vector
3μl 10x
2μl dsDOA
19μl H_2O
2μl vector

Into HE DH5α 11-23-92
11-30

Vector Control onto X-gal

Probe synthesis 11-25-92
1μl/100 = 261 KCPM
Added to bag 6:00 p.m.

11-23-92

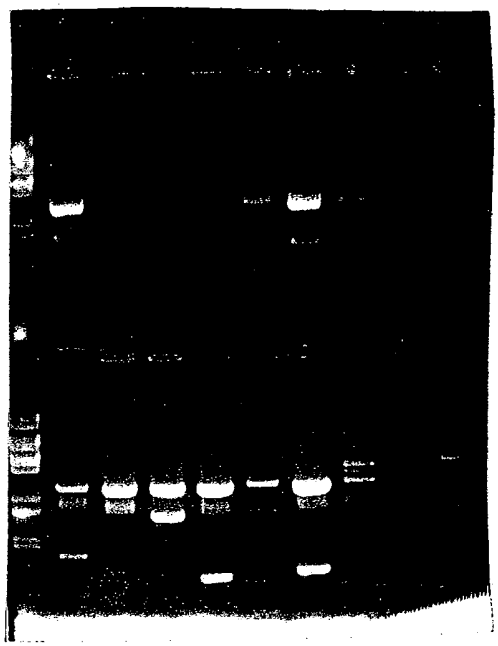
Of the 18 picked on 11-21
EcoRI-HindIII digested

1, #3, 5, 7, 10, 12, 13, 16 all saved for further characterization

Should be probed with 26S specific probes.

∴ Set up grids for colony lift

λ BSHE2
Eco/H3
1-19



Prepare H3-R1 frag from EEE-9
& R1-H3 from E. coli BE

Put 1, 2, 5, 7, 10, 12, 13, 16 into JM105

10-18

Since #1 may be a mix it is also streaked out on an X gal plate

#236 to page 81

Probe prepared with both fragments.
Labeled using BM kit with BRL Klenow
μl = 436 cpm/μl effluent

Boiled and onto filter 11-25-92 noon;
Washed 56°C, 2 buffer changes 8:30 11-26
GM Sup some are hot,

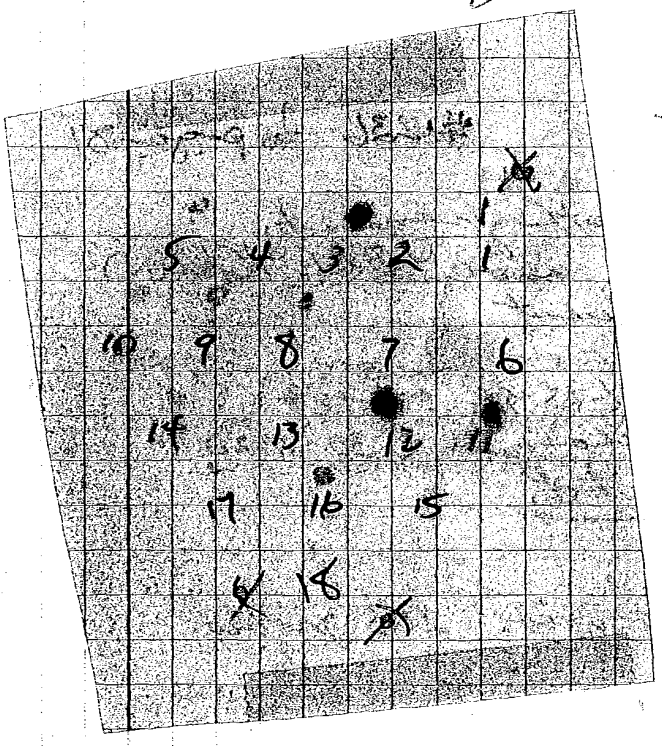
11-26 #3, 11, 12 ~~3, 11, 12~~ are hot when probed with 26S probes described above.

∴ Cut 3 with R1, H3 individually - does it extend upstream similar.
12 must extend upstream because it lacks R1 - H3 which at 1576 and 2600.
∴ Put ~~8, 9, 10~~ into 105

Freeze in following 5 lots.
ERP = random primed clones in pTZ18 Hind III

#1	233	~~ERP-1~~ ERP-1	
3	234	ERP-3	Desired 18, 17, 15, 14, 13
7	235	ERP-7	11, 10, 9, 8, 6, 5, 4, 2
12	236	ERP-12	
16	237	ERP-16	

In order to determine upstream limit, digest 3:12 with Bst E2 Nco1 and Nhe1.
If extra one present, do double digests to determine extent
Bst E2 at 130 high
Nco1 at 997 high
Nhe1 at 364 med.
Started 2:15
One may be unstable to continue work.
probably #3



Blot denatured on page 55.

Start a mapping of #7, 3

Is #7 EEE or something else? - no hybridization
7.35 kb

to page 61

Western cDNA cloning 11-23-92

Remaining RNA reprecipitated
Do 1st & 2nd cDNA Synthesis as outlined on
page ~~3842~~ #1 = oligo-dT
#2 = random primed,

Large pellet of material

Resuspended in 40µl H_2O - Remainder frozen in cloning
Use 10µl each reaction Magnet box - 20µl -

	Random Primed	Oligo-dT primed
t_0 -	17755	17325
t_0 +	388	791
t_f	18055	4990
net cpm	17667	3998
% inc.	0.044	0.023
nM inc.	19.8	4.6
µg cDNA	6.53µg	1.51µg cDNA

Why so deficient?
Compare size distribution on denaturing gel,
Probably too much primer -

Precipitated overnight!

After 2nd strand synthesis and spun columns,
Effluent was frozen O/N.
- 5µl dATP added ✓
- Volume O = 11µl E = 10µl
10x added 12.5µl 13µl

1µl T4 DNA Pol - 5min
37°C, 30'

	cpm remaining	% of original
5µl EDTA ✓		
O/N extract ✓	1µl - random = 4145	0.467
Spun column	1µl - oligo = 720	0.360
Etoh ppt		

WEE cloning
After spun column, effluent is EtOH ppt'd.

① Insertion into vector

pTZ18 Hind 2

New vector prepared by
- Hind 2 digestion - clean single band
- EtOH ppt
- T4 repair
- ØOH
- Spun column
- Adjust to 50mM tris, 1mM $ZnCl_2$
  ↳ note that no $MgCl_2$ was added
- 2µl ~~Ø~~ CIP
- ØOH
- EtOH 500µl-TE, 60°C, 2'

OD_{260} 0.280 = 14ng/ml

Suspend CDNA's in ~~20~~ 40µl H_2O
- Fractionate 20µl on 1% gel
- Ligate 5µl of remainder to Hind II - pTZ19 ~~pvu~~ ÷ 5 to pUC 13 2 ng/l
- Run 5µl of each on analytical AGE to determine distribution of label.

11-16-91 10:00 am Ligation

Sample	Insert	Vector	10×	H_2O	Lig
pUC-Control	-	3	3	22	2
pUC WEE	5	3	3	17	2
pTZ-C	-	2	3	23	2
pTZ-WEE	5	2	3	18	2

> Only using random primed material
Remainder of CDNA stored in −20°
as $R_0 \div O_0$ = Random ÷ Oligo-dT primed.

to pg 63

11-26-92

Purification of intracellular RDP
RVF control

- Vero cells infected with ZH548 RVF, T-150.
- Incubated 14 hrs, 37°C.
- cells scraped into cold HBSS and pelleted
- cell pellet vortexed in 1 ml 20mM tris pH 7.5, 50mM KCl, 5mM MgOAc, 1% NP-40
- Microfuged 1 minute
- Layered on top of 10 ml 32% CsCl in 20mM tris pH 7.5 5mM MgOAc
- Spun at 38K, 20°C, 18 hrs.
- Visible band collected and pelleted at 40K, 4 hrs.
- Pellet extracted and ethanol precipitated.

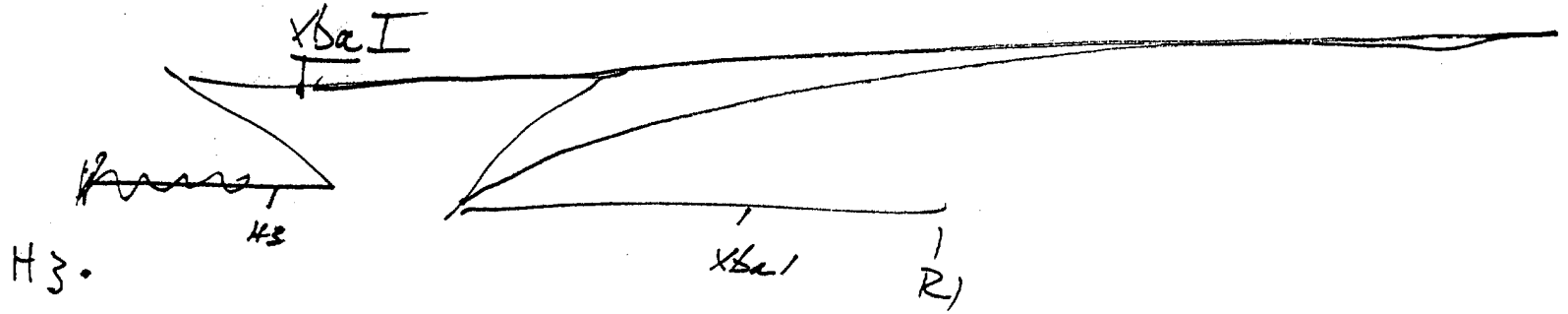
Note - gradient could probably tolerate 2-4x this # of cells

12-2-92

RDA pelleted
Suspended in 30µl H_2O
10µl run on Hg Gel. mutiral 1.2%.

lane	description
1	Ladder 3ug
2	EG RDA - 60µl
3 PEG-VERO	~~RVF EG~~ CsCl pellet - 5µl of 50 c H_2O
4	RVF RDP RDA. 10/30 - clear banding detectable
5	RVF CsCl pellet - 60µl

V_o = 80V
i_o 52
t_o = 11:25



H3.

600bp

ERP Cloning extended from page 56

ERP-3	R1	Nco1 (may not cut)	BstE2 — Nhe1 released identical Nhe1 fragment all cut single sites ca 12
	H3	Nhe1	

Lack of R1 & Hind3 sites indicates should extend upstream.

ERP12 Nco1 - may not cut
Nhe1 - release 0.6 Kb fragment
BstE2

~~Suggests that 12 extends further upstream than 3~~

The results are contradictory. Can only be rectified if 3 site further upstream than 12 and if 3 is ~~upstream~~ upstream of the Nhe1 site. However this leaves a target sequence for the E.C. probe of less than 300 nucleotides.

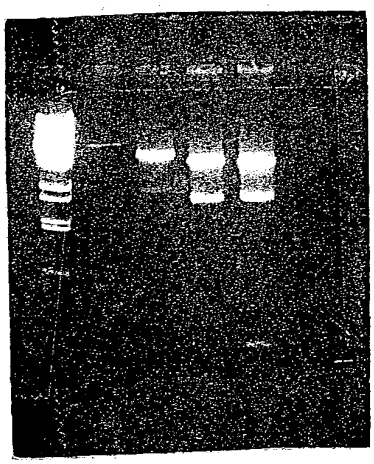
~~Cut both with Sma1 - predicted site present at 1696~~ Also cut 12 with Eco R1

Both apparently extend appreciably upstream, 3 may extend >2Kb
~~Neither digest suggests that~~

Map by R1/Nco digestion - 0/0 pTZ18 Hind III
Since both apparently lack the R1 site at 1596, should indicate orientation and sequencing approach

Also prepare #3 for additional probe synthesis when mapped.

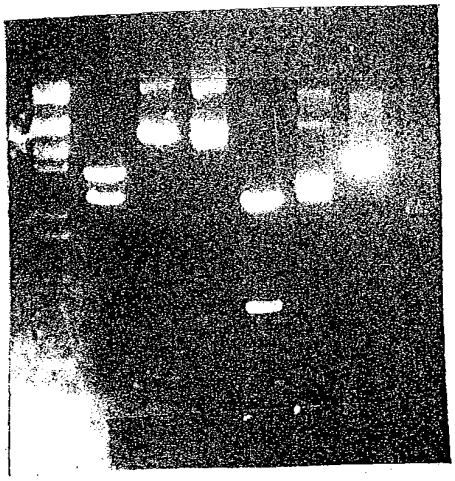
11-27-92



λ
ERP-3 R1/N2
ERP-12 R1/N
ERP-7 R1
ERP-7 R1/

Better mapping obtained with
R1/BstEZ cuts

1-28-92
Sequencing of #7 failed to indicate any homology to known sequences. Not surprising - saved for later at ERP-7.
Repeated cuts of ERP-7 indicates that it is ~1kb in length.



ERP-3 Mox
ERP-7 Mox

λ
-3
H3/R1
Nhe 1
-Mo 1
-7
As
Above.

10

Ø is also linearized by Bam H1, xba I, Kpn I and Acc I
No phe b labs

Therefore redo the sequencing of ERP-7 after ON denaturation of 8 ug of plasmid

-Also linearize 15 ug with ~~Kpn~~ I xba
-EtOH ppt
-Digest with xba I
-Exo III deletes.

to page 65

Western Parbo Synthesis

4µl RAA – 4°C, 1min
4µl H_2O
2µl Random primers
1µl control
5µl dNTP-dA
2µl RNAsin
5µl ~~3'~~-dNTP
2µl RT

5'RnT
1hr 42°C
5µl EDTA
4µl TE ^
3µm column
Incl effluent to LSC = 195 Kcpm

PCR reaction - Using WEE-1 WEE-3

2µl dNTP
62µl H_2O
10µl 10x
1.5µl 100mM $MgCl_2$
WEE-1 4µl (25µM)
WEE-3 12.5µl (8µM)
CDOA 5µl

Terminated at this point!

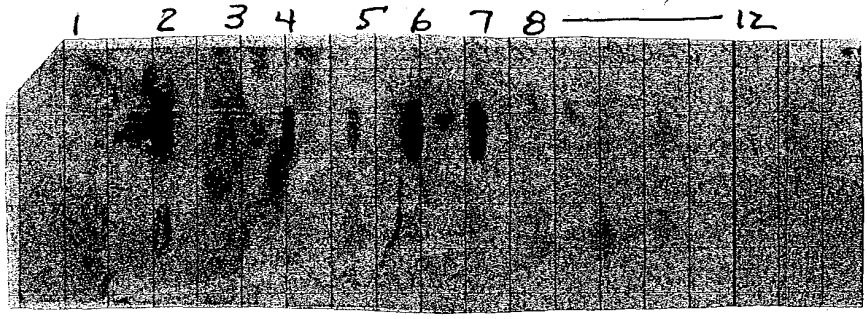
from page 62

Re cloning

Although ERP-3 hybridized strong to probe made from EEE-6 & EEE-E, sequence from both families was unrecognizable.

∴ In order to determine specificity of clones, analyze in slot blot format.

Controls -
1 - pT2.19
2 - pEEE-6
3 ERP-1
4 ERP-3
5 ERP-7
6 ERP-12
7 ERP-16
8 Additional Eato clones
9 5-20.
10



Blot #1
Probe E.C 6 cDNA

Do blot in duplicate
Probes #1 with E.C 6 probes
#2 ✓ cDNA made from ~~OOH~~ CsTFA extracted material

Protocol - Hybond NE
- ~~Boil~~ Tag DNA in ~~20 µl~~ 100µl H_2O
- ~~Add Dyl 2M Tris~~ Boil 1 minute
- 37°C, 20' Quench, Add 100µl 20x SSC
- ~~Apply~~ to blotter under suction
- Apply to blot pre-wet w/ 10x SSC
- ~~Transfer~~ to blotting paper saturated with 100mM NaOH

- Transfer blot to denaturing soln — 1 min
- Neutralize - 1 min
- Blot excess buffer
- Air dry

- Dry on 3MM Paper saturated w/ 100mM NaOH, 20 min
- Rinse briefly in 5x SSC

Blot Preparation

Rewet membrane in 10x SSC

① Dilute herr DNA or Sub mini prep DNA in 100µl H_2O
② Boil - 2 min
③ On ice
④ Add 10µl 20x SSC
⑤ Apply to membrane under vacuum
⑥ Transfer membrane to paper saturated with 0.5 M NaOH, 1.5 M NaCl, 5 min
⑦ Transfer membrane to paper soak with 1.5 M NaCl, 0.5 M tris - 1 min
~~⑧ Wash briefly - 0 seconds -~~
⑧ Allow membrane to air dry.

⑨ Fix blot by placement on filter paper saturated w/ 400 mM NaOH - 20'
⑩ Wash briefly in 5x SSC
⑪ Start pre-hybridization

Probe preparation
Using Frags described on page 55

4µl each frag

	8µl ✓
Boil - 10 min	
Quench	
3µl Reaction mix	3 ✓
1µl BRL Klenow	1 ✓
1µl dCTP-dA	1
4µl ^{35}S	4 ✓
13µl H_2O	13 ✓ 1:05
	30

to page 67

NAK cpm/µl effluent

PE6 cloning cont'd. 11-30-94

cDNA probe PE6

- vRNA prepared by Ø-OH extraction
- Pelleted & dried
- Suspended in 30µl H_2O
- Remove 5µl for cDNA synthesis

5µl vRNA ✓
13µl H_2O ✓
1µl random primers ✓
65°C, 2' ✓
Quench ✓
16µl cocktail ✓
2.5µl dNTP-dA ✓
2µl RNAsin ✓
4µl ^{35}S ✓
2µl RT ✓
+3µl H_2O
5min RmT, 1hr 42°C. ✓

5µl EDTA
45µl TE
1µl to filter
Spun column → 12.5 Kcpm/µl effluent.

Discard

Instead

Digest 15µg of pRP3 with EcoRI & HindIII and isolate 4.1 KB insert

Prepare probe and probe second slot blot of cellular & viral RNA's.

5µg in 50µl reaction

Heat for

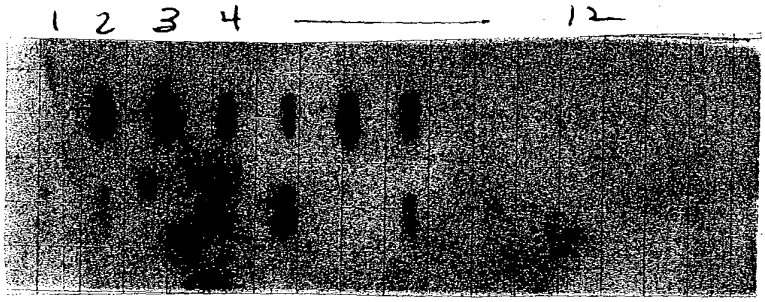
Second slot blot

Reprobe with cDNA (^{32}P) radiolabelled with the using CsTFA virion RNA as template

Prepared as on page 63

1µl = 821Kcpm - very high channel 2

Blot2



13 — 24

∴ Although the terminal sequences on ERP3 do not match anything, it appears to be virus specific

One last blot-

Probe cellular extracts with ^{32}P-cDNA prepared from ERP-3

15ug digested with EcoRI + HindIII.
Run out on gel
Slice lane a second time
elute & label

RNA Blot
1 pT218
2- ERP-3
3- RNA ladder
4- E6 RNA
5- E-1cRNA
6 - E-RNA - phenol extract
7 - λ DNA.

Suspend samples in 100μl H_2O
Boil #'s 1, 2, 7
5min
Add 100μl 2OxSSC to each sample
Apply to blotter

	G,E Probe	cDNA probe	vCDNA - Northern blot
pT2	—	—	—
EE-6	+	+	
RP-1	—	+	
-3	+	+	+++
-7	— ?	+	
-12	+	+	
-6	+	+	
E6 RNA			—
RNA ladder			—
RNA - E6 - EEE			±
E6 vRNA			+
λ DNA			—

Blot ~~Purification~~ Preparation — from page 68.
Northern Slot Blot

To determine if the contradiction between the hybridization + sequencing data of ERP-3 is due to contamination of the probe or the substrates used to prepare the probe.

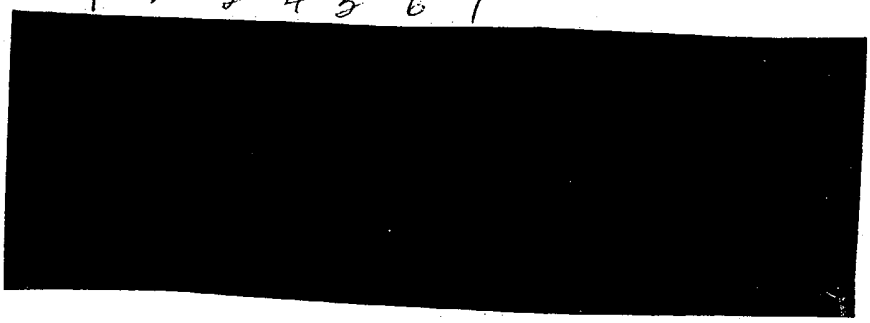
	Slot	Description
—	1	
++	2	PTZ18 1ug
—	3	ERP-3 1ug
—	4	RNA ladder 3ug
+	5	EG RNA - 100µl
+	6	PEG-I.C. RNA - 100µl
—	7	PEG VRNA -
	8	λ DNA - 1ug

All RNA's are mixed as determined by AGE

Membrane - HYBOND N not HYBOND N+

- Dilute all samples to 100µl
- Boil 1, 2, 7 for 5 minutes
- Place all samples on ice.
- Add 100µl 20x SSC to each
- Apply to blot
- Air dry
- 2 minute exposure to U.V. light

+++
1 2 3 4 5 6 7



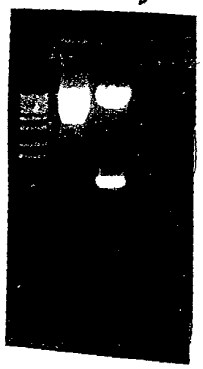
Prior to full scale sequencing of pERP.2 additional ~~digests~~ restriction mapping required

λ Nco

λ Bam / R1 / RV / Sal / Sca / Nhe / Hind 2 / Sty 1 / Bgl 2
-Lvo - - +

Xba ? release entire fragment
Sma 1

Kb
- XbaI
- Sst1



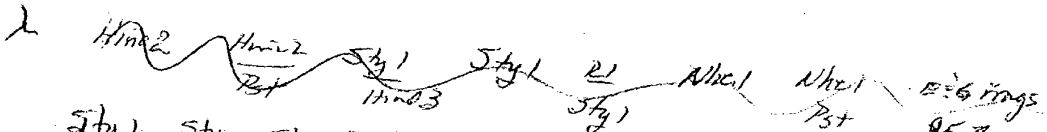
Map one cutters with respect to Hind 3 site; R1 site - probe for more walking

H3/Bam

H3 RV
H3 Sal
H3

Prior to sequencing, digest & Southern blot ERP 3
Duplicate gels, vertical 1% TBE

35
λ



Sty 1 | Sty / R1 | Sty / H3 | Pst | H2 / Pst | H2 | Nhe / Pst | Nhe 1

on minigel 12.5ul/well

11-5-92

Attempted PCR of 3' end of Chik virus A.

Reverse transcription - using remaining Vaccine + parent transcripts protocol of page 43

20 µl RCA ✓
42°C - 2' ✓
3 µl oligo-dT ✓
10 µl 5mM dNTP ✓
2 µl RNAsin ✓
32 µl buffer cocktail ✓
24 µl H_2O ✓
3 µl ~~^{3}H-dCTP~~ ^{35}S-dATP ✓
4 µl RT ✓

2 µl samples

	V	Parent
t_0 −	63265	695 666
t_0 +	2299	2637
t_f +	3097	5294

DH 15'

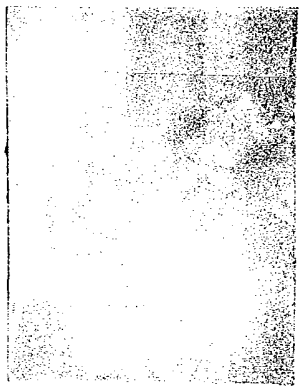
10 µl of each to PCR Reactions

A-As a control - use Gap primers on both

3' primer were improperly synthesized - polarity reversed

∴ Do Parent PCR -

(A)	CHIK E2	dNTP	2 µl ✓	95°C - 45"	
(B)	GAP	H_2O	66 µl ✓	45°C - 1 min	
(C)	E2+ - GAP2	10X	10 ✓	72°C - 2'	λ
		$MgCl_2$	15 ✓		A/B/C
		Taq	0.5		
		Primer 1	5		
		Primer 2	5		
		cDNA	10		



1092	E2+	(5)	5	✓	Discard Mix A, C
1575	E2-	(5)		✓	
3448	Gap1	5		✓	
910	Gap2	5	5	✓	
ected product		0.5Kb	0.5Kb	2Kb	

D-D

Cloning of CPgag

Suspend ppt'd in 30µl H_2O
Remove 15µl
Save the remainder at -20°C
Slot #8, -20°C
15µl + 3µl 10x high salt
1µl H_2O
1µl Eco RI
37°C - o/n.

Repeat the gag PCR on the tiny amt of RT product of vaccine RNA

d-NTP	2 µl
H_2O	66
10x	10µl
$MgCl_2$	1.5
Taq	0.5
Primer 1	5
Primer 2	5
cDNA	10

Predicted fragment was produced.
$CHCl_3$ extracted
EtOH precipitated

Suspend in 30µl H_2O
RI digest 15µl.
Save remainder at -20°
Slot #9, CV gag PCR product

After Eco RI digestion of CP gag, 3 well defined fragments were apparent Eluted as CPgag 1, 2, 3 with decreasing size.

12-7-94

pT2.19-Eco RI (53ng/µl)

Sample	Amount	Vector	10x	H_2O	Lig
gag 1	5	3	3	18	1
gag 2	5	3	3	18	1
gag 3	5	3	3	18	1

from page 72

12-7-92
gap PCR Product from Vaccine
digested with Eco RI
2 bands
Into pT29 Eco RI
Into JM105's 12-8-92

12-10-92
Eco RI/Hind 3 digest of
ten colonies indicate that
~ 300bp frag came out of
#7. Faintly visible on
photo. Actually, fragment
was present in all ~~10~~
colonies.

→ From Vaccine Strain

Culture refed 0/N, part frozen and
prepared for sequencing from both ends.

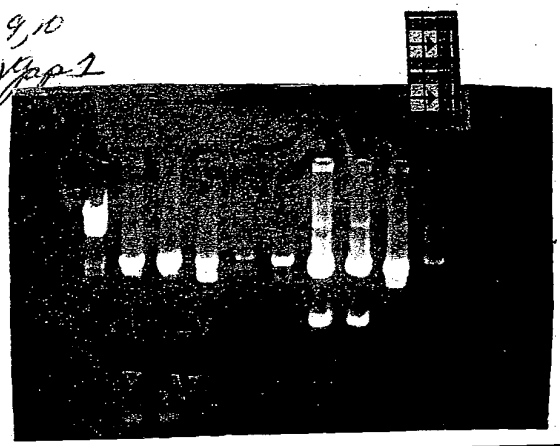
Stored in slot 238 CVgap1

Sequence completed 3-25-93
Presence of Eco RI site prevented
Complete sequence of gap.

Need to get other band to complete sequence

4-3-93
Additional clones picked and miniprep'd.
Digest with Eco RI/ Hind 3

1, 3, 4, 5, 6, 8, 9, 10
CVgap1



#6 has smaller frag than
CVgap1
#8 has larger - "undigested" frag.
Prepare ⊘ for sequencing of
both.

4-3-93

BASED UPON HOMOLOGY TO ONN & SFV, THE COMPILED SEQUENCE TO THIS DATE MAPS TO

	CHIKV26S		pCEV-5
ONN	11190	11210	
SFV	10980	10980	

∴ ~ 20 nucleotides remain undetermined.

∴ Do the sequence indicated on previous page. ✓ Prep

11242 11182 4-12-93
11242 10960

Gap 1 F left to repeat

Some confusion around internal R1 site of CVgap2R Phosphorylate and
∴ clone entire fragment and sequence
4-16-93 in. into ~~pTZ19~~ puc 13 SmaI

Plate 10^{-4}, 10^{-5}, 10^{-6} dilutions of Chik library 38-42 ∴ probe with pCEV5 frag to locate 3' end clone.

Redo reverse sequence of CV gap 2 with α-ITP analogue to clear up sequence at internal R1 site.

→ Single clear positive picked for mini prep ∴ R1/HindIII cut ∴ ~ 200 bp fragment

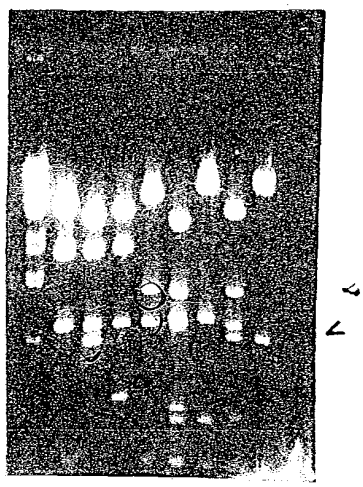
Ligation

Sample	Vector	Insert	10×	H_2O	Lig	
~~-~~	3	0	3	22	2	puc13 SmaI
+	3	5	3	17	2	into SureCells - 3µl

~100 colonies each

- Library Efficiency 1 x 10^6 cfu/µg from 5µg

Analysis of pERP-3 From 70



Bkd Head to Nutran
Voltage Adjustment 40
λ_0 = 250mA

Subclone Nhe frag to pTZ18
page 79

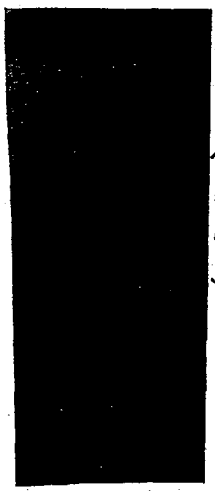
11-9-91
During the course of blot preparation, sequence compilation of pERP-3 showed by limited homology with VEE, that 3 does in fact represent the E ns genes.

In order to extend the walk another step, prepare to ~600 bp Sty1, Eco R1 fragment for probe preparation
10ug digested with 15u R1, 15u Sty1

Try Hind3-Sc digest releases intact Fragment → NO! Wrong end-vector is pTZ18

λ
Acc 1
AVa 1
Sst 2
ported?

Double digest
ERP3 with
H3/Ava 1 Whitey cells
H3/Sst 2 and additional
H3/Acc 1 frag to use for probe?



O/N 12-11-91

Result re: conclusions. ERP4-2 12-12 ~ 0

R1/H3 digestion yield ~0.7Kb fragment

Probe preparation

- ~~10~~ul/20 boiled 10'
- Quench
- 3ul reaction mix ✓
- 1.5ul 5mM dATP-dCTP ✓
- ~~10~~ul ^{32}P-dCTP 11-27-92
- 4.5 ~~5~~ul H_2O
- 1ul Klenow

- 2hr hybridization in Amersham Rapid hyb. buffer
- Wash 1hr in 0.2xSSC
- Radiograph O/N.

12-14-92

8 additional positives picked for minipreps.

λ
1-8
R1/H3
digest

Are these real? ?
How to tell if they're really NS
12-19-91

Slot blot: reprobe with 12-2 frag - on Hybond N
1 - pTZ18 1ug
2 ~~EEE-6,E~~ - 1ug each → 1-25-92 actually correspond
2-10 clones 1-8 to 5'end of 21S + 3'end of nsp genes
11-ERP3

- Suspend 5ul of minipreps 1-8 in colony lysis buffer
- Spotting to slot blot
- Wash with colony neutralizing solution
- Dry & fix

Prepare prep as above ~~35~~-dATP ✓DuDOA ✓Boil, Quench ✓~~5~~ul dATP ✓5ul 35S to page 8

12-10

SEQUENCING ERP-3

Nhc : What enzyme

Sample Designation

Previous samples not noted here

		DATE	Discarded ? / or Frozen	
3-1	1	12-10 ✓	Dis	
3-2 3	2	12-12		Frozen 239
6-1	3	12-10 ✓	Dis	
6-2	4	12-12	**Discard recommended pt 2**	
7-1	5	12-10	Discard X 12-11-92	
7-2 3	6	12-12	~~Discard~~	Frozen 240
8-1	7	12-10 ✓	Dis	
8-2 3	8	12-12	Discard 12-12	Frozen 241
9-1	9	12-10 ✓	Dis.	
9-2	10	12-12		Frozen 242

12-1	D	+
12-2	E	+ Discard 12-11-92
16-2	J	+
18-2	M	+ Discard
6-2	13	+

N	14-3	14-4 + 15-4 dient Discarded
O	15-3	~~minimal~~ grow after injection — O.D DNA prep looked a little dirty - would
P	16-3	- Run short
Q	16-4	Discard

12-12-92 Plates 10-12 15 discarded. All colonies picked. 16 discarded 12-15-92

Run short gel of ERP 3-3 #2 ✓
ERP 7-3 #6 ✓ 12-13-92 Discarded
10-3 #P ✓

Long of 9-2 # 10

12-12-92

R	~~5-3~~
S	5-4
T	5-5
U	6-3
V	6-4
W	6-5
X	4-3
Y	4-4

A 10-1 - 12-13 Discard
B 11-3 12-13 Discard
C 11-4 12-13 Discard
D 12-3 12-13 - Frozen ERP12-3 #243
E 12-4
F 13-3 12-13 Discard > 12-14-92
G 13-4 - ?
H 14-5 12-13 Discard Slot 244
~~I 14-6 Frozen ERP14-6~~

12-13-92

J 0-3 Discard
K 0-4 new ⊕ preps
L 1-3 Discard
M 1-4 "
N 2-3 Discard
O 2-4 "

Include G 13-4 also

P 13-1 Discard
Q 13-2 Slot 246
R 13-3 Slot 247
S 14-7 Repeat 12-18-92
T 14-8 Discard
U 14-9 Discard
V 13-4
W 14-10 Discard

12-20-92

X 13-5 Trying to extend 5' end of ERP-B.
Y 13-6
Z 13-7
A 13-8
B 13-9
C 13-10 4-CX-Z on gel

from page 25

Subcloning Vhe 1 frag from pERP-3.

10ug pERP-3 digested O/N with 15u Vhe 1 in 60ul

add 3ul 5mM dNTP
6u Klenow
37°C, 30'
3ul EDTA

AGE
elute fragment

Ligation

Vector pTZ18 HindIII Z	2ul ✓
Insert	5ul ✓
10x	3ul ✓
H_2O	18ul ✓
Ligase	2ul — 2u —

lmT, O/N

~~Inoculate JM105's~~ JM105's
~~DTT~~

12-15-92

10 colonies picked for minipreps.
N-1 → N-10
All were recombinants
#1 boosted for maxi prep
frozen in slot 245
ERP-Vhe 1

Maxiprep 12-17-92
Remainder of mini
Digest w/Xba 1 — O/N.
EtOH ppt
Kpn Digest
Exo III delete.
6 time points.
Repeat 1-7-93 1-8-93 After ACE

~~Sequence #1-6 1-7-93~~
EtOH ppt
Xba in 20ul

50ul phamid = 12ng ✓
10ul 10x React 4 ✓
36ul H_2O
1ul ~~BSA~~
3ul Kpn — 30u

20u additional added

9:30

20µl digest
62µl H_2O
10µl 10X
8µl ExoIII

15' time points.
Only a smear is obtained.
∴ Prepare new stock of plasmid
Sequence miniprep both directions

1-12-92
New maps started - #94

1-18-93
Start deletion protocol.
Digest Mrg with Kpn.

from page 76

Walk to extend EEE-map

cDNA library probed with #9 > EPR12-2
8 colonies identified

~~from page~~

When additional clones are probed —
Best signals obtained from
2, 4, 7, 8
Surprising that 6 does not light up.

Why do E.C. 6 fragments in slot 2 continued to light up? ~~—~~ DI, management?

Freeze these cultures in temp storage box

Sequencing #12 from page ~~236~~ 56 Stock 236

- Cut with EcoRI
- EtOH
- Blunt end with Klenow + Thio-dNTP
  - 2.5µl 10x High Salt ✓
  - 2µl Thio-dNTP ✓
  - 20µl H_2O ✓
  - Vortex ✓
  - 0.5µl — 3U — Klenow ✓
  - 37°C, 20' ✓
  - 65°C, 20' ✓
  - Cool + Add XbaI ✓
  - 5?
  - 10µl 10x
  - 5µl Exo #1
  - 20" time point

1-10-93
ACE

only samples 5-12 were sufficiently resolved.
XbaI digest must not have been complete.

Process and ligate
Transform JM105's.

18
1-16-92

Sequences new autogel

Pick's 2 additional colonies from time pts 5-R.
Grow O/N and prepare using Cartridges
for sequence on 1-19-92

Additional work.

ERP 12

Deletions of ERP-7 Prep #92

① Ligation with Kpn I 20ug
② Ppt
③ Digest with BamHI 1hr at 37° in x-ba site at position ~250

1-20-93
14 time pts harvested
Repaired
Ligated w/ Xba
Used to JM105 — new batch w/ pT2 19 control.

5μl 10x High
40μl H_2O
5μl BamHI 705

Some mold contamination
Visible colonies picked
7-1, 7-2, 7-3, 7-4, 7-6, 7-7, 7-8 Plates then discarded
Start ⊕ but also do minipreps before sequencing
EtOH ppt ligation and re transform.

Minipreps suggest that deletions are OK
but do not fall into line with time of Exo
treatment.

Prep 93 — JM 105 again

Preparation of H3pTB.
pTZ18 lacking H3 site.

- 5ug digested with HindIII - 100µl reaction
- 5µl 5mM dNTP added
- 1µl Klenow - blt
+37°C, 20'
- 65°C, 15'

3µl of digest
+3µl 10x ligase buffer
23µl H_2O
1µl T4 ligase
RmT, 2 hrs

Transform JM105
Plate on X-gal

All white colonies

Pick 5
Digest with HindIII
Digest with BamHI

Colony #1 frozen as H3pTB
Slot 249

Sequence HindIII site changed
to ~~GAATC~~
△
AAGCTTC
△

Cloning EEE 5' terminus 1-22-97

① Synthesize first strand as on page [illegible]

10µl rRDA (X = which RDA sample.
5µl random primer - X
10µl 5mM dNTP - X
5µl RDAsin - X
32µl buffer cocktail + X
2µl H_2O - X
4µl RT X
3µl ^{35}S dATP X
5' RmT, 42°C, ~55'

Cocktail
20µl 1M tris pH 8.3
28µl 1M KCl
40µl 100mM $MgCl_2$
40µl 100mM DTT

2µl samples

		net	%	nM inc.	µg cDNA
①	E_0 - 598300				
②	t_0 + 201712				
③	t_f + 20400	18688	0.031	6.2	2.04µg
④	W_0 - 517660				
⑤	t_0 + 2884				
⑥	t_f + 15338	12454	.024	4.8	1.58µg

5µl EDTA to each
Two WEE
Add 100µl TE
Phenol extract
133µl 5M NH_4OAc
EtOH - -70°C, o/n. to page 88

To EEE
3µl 10N NaOH
2mT/o/n
1-23-97 50µl 1M tris pH 8.3 added
15µl 1N HCl
+15
+15 → pH ~ 6.0
Dow 2x
25µm columns 100µl each
Combine eluate - counts by GM
EtOH ppt, 23µl NH_4OAc Dried & stored at -70°C
dC-tail

TdT Standardization

1x Buffer 200 mM K+ cacodylate pH 7.0
~~4mM MgCl2~~
~~1mM BME~~
~~1mM dCTP.~~ 10mM $CoCl_2$
1mM DTT

100mM K cacodylate pH 7.2

BRL formulation

ΦX DNA 3.5×10^6
5386 bp

λ 48,502 bp MW = 31.5×10^6 daltons.

Using an oligo as substrate

Using forward primer in slot F-1
18-mer
Concentration = 338 ug/ml
= 63.7 μM

Set reactions to contain
~~20~~ 20 pMoles of 3' ends
∴ Dilute oligo 1:63 = 10 μM
= 10 pM/μl

Reaction #
1 – 1mM dCTP
2 – ~~2~~ 50 μM dCTP
3 – 200 μM dCTP

Reactions mix

	1	2	3	
~~10~~ μl	5x TdT			x
5 μl	^{3}H-dCTP			x
1 μl	oligo			x
oligo				
10mM dCTP	5 μl	2.5 μl	1 μl	x
H_2O	27	29.5	31	x
	3 μl TdT			x
	= on ice	✓	✓	
~~50~~ μl samples	0 ✓	15	30	45 60

TdT Testing from page 86

Sample	Cpm	Net	%I.M	dCMP inc	dCMT/3'end
1	172270				
1-0	426				
1-1	4239	3813	.022	1.1	110 — Use this reaction
1-2	7804	7378	.042	2.10	210 3 minutes
1-3	12496	12070	.07	3.5	350
1-4	14845	14419	.083	4.15	415
2	220978				
2-0	566				
2-1	5375	4809	.021	.525	52 — Use this time point.
2-2	6366	5800	.026	.65	65
2-3	7570	7004	.032	.8	80
2-4	7616	7050	.032	.8	80
3	190084				
3-0	561				
3-1	4812	4251	.022	.22	22
3-2	5021	4460	.123	.23	23
3-3	7410	6849	.037	.37	37
3-4	5186	4625	.024	.24	24

Tailing cDOA.

Suspend cDOA in 32 µl H_2O ✓
5µl 10mM dCTP ✓
10µl 5x TdT Buffer ✓
~~37°C, 2 min~~ Ice ✗
3µl TdT ✗
37°C. 8 min.

5µl EDTA ✓
150µl TE ✓
EtOH ✓
EtOH ✓
2nd strand 50µl reaction

5µl oligo-dG 12-18 to 100 ng/ml ✓
5µl 5mM dGTP ✓
5µl Med Salt Buffer ✓
34µl H_2O ✓
1µl Klenow – 6U
22°C. 30' 4:45

2nd strand of WEE
Sample warmed to RmT
Pelleted 15
EtOH removed and pellet washed w/ 70% EtOH

2nd strand synthesis as on page 44

✓ 1.75hrs 12°C
✓ 1hr 22°C
✓ 5µl EDTA
✓ 150µl TE
✓ ~~6~~ OH
✓ EtOH
T4 Blunt end
415µl H_2O ✓
5µl 10x T4 Pol ✓
2.5µl 5mM dNTP ✓
1µl T4 Pol 7d
~~37°C~~, 20' 5:35

5µl EDTA ✓
45µl TE ✓
~~6~~ OH
Spun column
6136 cpm/µl = 613,600cpm / 622700 initial
15 µg CDNA remaining

EtOH precipitate and ligate
Suspend pellet in ~~20~~ 30µl H_2O
Ligate in 2 batches
1 to pTZ 18 Hind 2 (T4, CIP)

Sample	Insert	Vector	10x	H_2O	Ligase
WEE-1	5	2	3	18	2
WEE-2	1	2	3	22	2
EEE-1	5	2	3	18	2
EEE-2	1	2	3	22	2
Vector	0	2	3	23	2
		✓	✓	✓	✓

1 to HB101

5' Cloning of pT6 — from page 67

5µl EDTA ✓
45µl H_2O ✓
OOH ✓
Spun column ✓

1µl → cpm 4428 cpm

= 1/42 800 cpm
Initial cpm – 934/400 = 2.0 µg

Adjust for T4 Repair

17µl pdDOA
12µl ~~10µl~~ µl 10× T4 buffer
10µl µl dNTP
2µl T4 PNK

37°C, 30'

= 967 ng remaining before T4 repair

Is this due to T4 clean up of non-ds cDNA ?

5µl EDTA
OOH
Spun column
1µl = 5300 cpm
= 2600 cpm/µl

~ 450 ng cDOA remain

10µl NH_4OAc
EtOH
-70°C

Ligation as on preceding page for WESTERN.
pT218 HindIII (T4 CIAP)

Ligate to HB101

Probe this & other library with ERP3 fragment
Nhe-H3 fragment.
Maybe significantly contaminated with nucs
200 ng pT219 digested with BglI and boiled with probe.

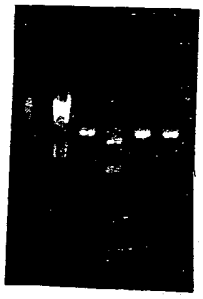
15µl eluted DOA
Boiled 10' Quench
5µl reaction mix
5µl dATP-dC
5µl ^{32}P-dCTP
23µl H_2O
30.5µl K/amon (3a)

pT219L added to column effluent & boiled

Added to rapid hyb buffer, 1:00 p.m.

One additional clone
ERP-C identified 2SI slot
Terminal sequences indicate that
it spans junction

Restriction cut



- Linearize 10ug with EcoRI
- Blunt end w/ Sculptclean
- Digest with XbaI

2-9-93 into TM105
2-10-93 some colonies from all except samples 2+5.
Start two/plate when possible

Sequencing ERP-1

Overlaps 7
∴ Digest with StyI - 20ug
Also Run R1/H3 control.

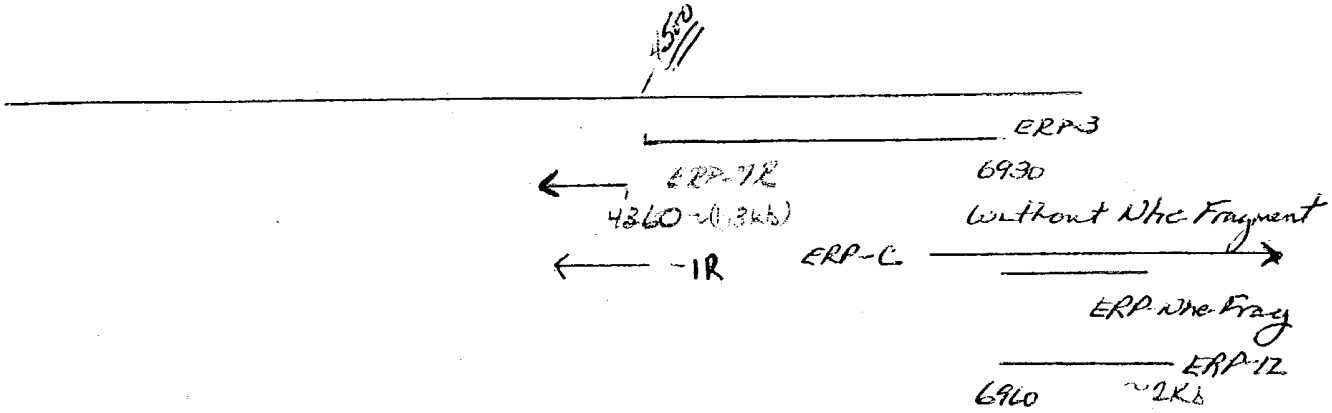
2-26-93
Too many colonies from 5' end cloning
Discard vector prep
~~Prepare new pTZ18 cloning vector~~
~~Digest 10ug~~
~~15ul 183 ng/ml~~

Ligate ECDA4 into new vector prep.
Ligate in old vector prep ✓
pTZ19 this 2 tube 18
(0.4ug/ml X ✓ X ✓

	Vector	Dna	10x	H_2O	lig	
Vect	4	0	3	21	2	1

EASTERN NS CLONE MAP

1 cm = 500 bp



Note - Sequence homology places -12, 7, 1 heading upstream at ~ 4000. AA shows actually heading downstream at ~ 6900

Because original ERP-12 sequence doesn't agree with sequences of deletions,

Redo Nhe I digestion of 12 DOA stock and compare with original map. Do deletions if correct.

Nhe sites are there. Do comparative digest of ERP-7 & 12 - do both have the same Nhe I sites no Xba sites.

∴ 12 looks like 3

The original ERP 12 sequence as entered in ERP-12R : -12F maps to the 5' end of the genome when translated but the restriction map looks like E-12P3

Do comparative digest.

215

← ERP-CF 485

ERP-7 sequencing from page 82

Photo is of EcoRI of minipreps
Φ started ~ 2:30

Sequence Φ
└ Trying to start nearest to digestion start.

1-29 Still yielded a large percentage of rearranged Φ.

1-30-93 Repeat using EcoRI, SdaI PstI & XbaI digestion

Purify additional insert for probe and probe original all libraries!
20ng = 5μl

1-31
Sunday
Replate all libraries for probing.

Probe Synthesis

① Scrape and plate original ERP library # 18 11-23
② Deblot & plate original Lig-dT Library #

Tufted and pre-hybridized O/N, my own buffer

Probe synthesis
5μl fragment slot 9 ✓
Boil & quench
5μl ^{32}P dATP ✓ Repeated 2-7-93
3μl Reaction mix - (B11)
3μl dTTP-dC
13μl H_2O ✓
1μl Klenow
30' Spun column

One additional clone identified 1-31-93 from previous ERPC clone

2-7-93

Deleting ERP-C

Including λ control

Digest with Kpn : ~~EcoRI Separately~~ 1.50 μ in
As a control ~~Also~~ Both give single cuts

- Use Kpn digest, XBa digest of the EtOH

Also ligate library
5μl ERP-7 21/113 frog Tube 91
Dil ~~2μl H₂O~~
0.8μl random primer
5μl 10xM
5μl dNTP-2A
5μl 35S-2A
0.5μl Klenow

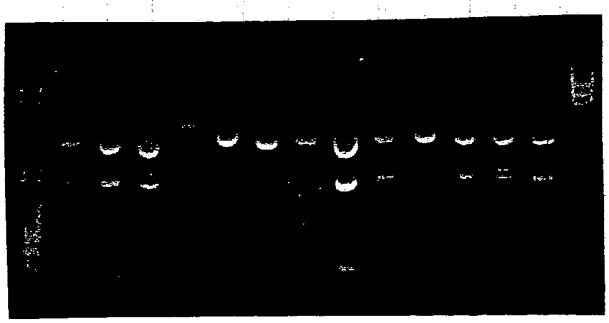
→ No new large colonies found!!! 2-10-93

→ Add 20μl 10x Exo
135μl H_2O
37°C - 2'
5μl Exo 3
20" time points.

2-8-93
Seems to have worked
Elute 0-5 electrophoretically
GeneClean 6-8.

Ligated and into JM105 2-9-93

12 picked for mini's. digest with PvuII



Sequencing of ERP-12

Digest 20µg with Kpn & 20ug with R1

Xba 1 seems suitable for second, still looks like -3.

Find the original d12

2-12-93 Prep 93

15µl + Kpn 1 3:30

20 + 3µl EcoR1 3:50

- Blunt end — in 5µl
- Digest with Nest ~1/100
- Dilute & delete

→ 4µl sample — to -70°C

- 3µl 5-dNTP
- 1µl — to Klenow — 5'

- 5H, 70°C, 15'

- 3µl Nco1 — 5'30 - 6'30

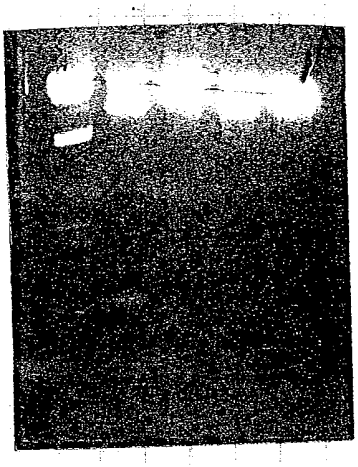
→ 4µl Sample — Bal to mixing 1

→ no evidence of Nco1 cut 1, must not be Prep 7

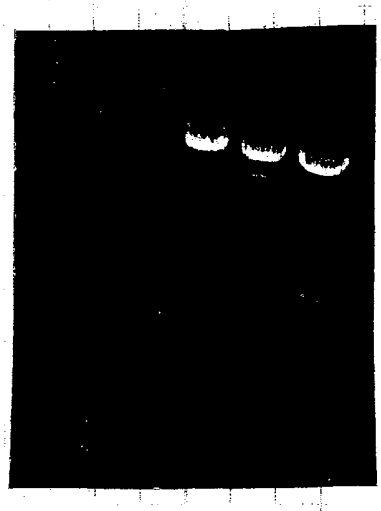
∴ Dilute to [illegible] and add Bam H1 — should be present [illegible]

Prep — 14 samples taken, -70°C, 15'

ERP-7 Xba
-12 Xba
-7 R1
-12 R1



-7 Nhe
-12 Nhe
12 Kpn
-12 Bam



Also start 2nd batch of R1 for 2° Xba digest after fill in.

Klenow Repair

50µl
+ 2µl 5-dNTP
+ 1µl Klenow
- 5min 37
- 70°C, 15'
- 2µl Xba 37°C 6'20

Sample taken before addition of enzyme runs differently than the others.

Much inert material

Slow rate of digestion — DOA excess?

2-14-93
R1 + Xba cuts repeated
30 second samples.
To control for MBD activity, will add
SS DNA as control to last sample.
— None available

Sequencing the gap in PEB-265. at

Up to this point the PEB-265 is sequenced to residues 3969 – homology to 3915 of EEEV-265 nt 3915

The EEE-1 sequences to continue from ~4220 to the poly A tract.

∴ To fill in the gap

3-5-93
- Digest EEE-1 with Eco R1 + Eco R5.
- Blunt end
- AGE + ligate
- In to JM105
- Sequence with forward primer.

Note - the computer indicates 2 RV sites, in addition to the large R1/RV frag, a ~350 bp frag should have also been present.
None seen on the gel.

Assembly of PEG-265. From page 42

2-2-93

Digest E265-Sal with Spl I (first) -55°C
then Sca I

pTZ18 Harvest 3.6 Kb frag

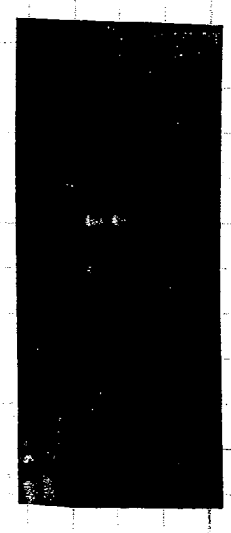
Digest EEE-1 with Spl I (first) -55°C.
→ Contains 3' terminus, then Sca I
Bam Site, pTZ19 Harvest 2.5 Kb frag.

HARVEST BY ELECTROELUTION —
Combine & ligate

1 —

Spl I cuts are complete
Sca I added without buffer change

λ
265 Sal
E-1
Spl I / Bam HI
digests

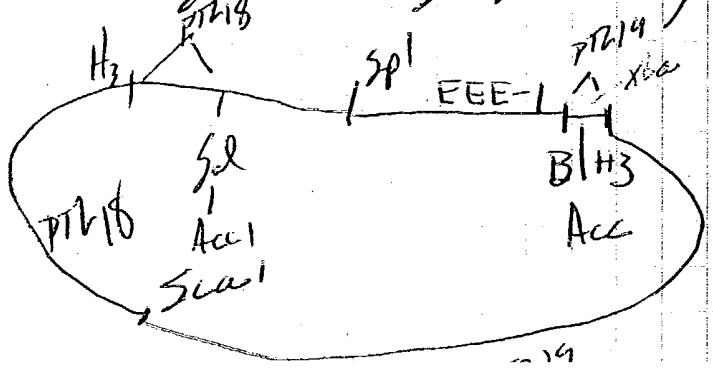


Both large fragments electroeluted, ethanol precipitated and ligated in equal proportions

Resuspended DNA's in 15 µl H_2O

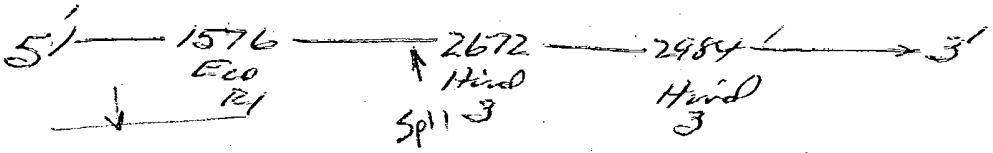
5 µl E-1 ✓
5 µl E265 ✓
3 µl 10x Ligase ✓
15 µl H_2O ✓
1 µl ligase ✓
Into HB101, 2-4-93

2-5-93
Many colonies appeared
Picked 12 for mini preps. + digest with R1/H3
No R1 was really necessary



See Sequence file 265-6

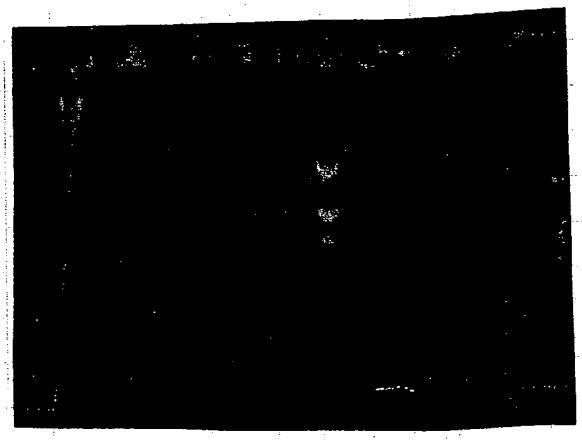
Predicted 265 fragments R1/H3



1576 1096 312 1000 bp

Appears that one fragment is not of the ~~pattern~~ predicted size.
How to map?

Hind III digestions #7
Spl I digest
Acc I 2164
Bgl 2 3052
Nco I 997
Sma I 1698



R1/Hind3 digests of 1-12
#7 grew much better in LB+Amp both the and the oth

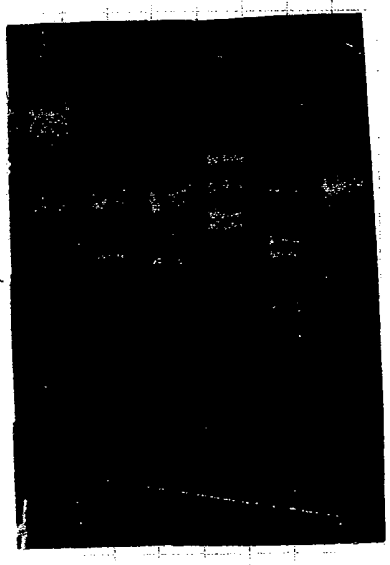
Hind 3 digest

2672, ~~1500 + vector~~ 312 + 1Kb + vector

Hind 3 / Bgl 2
2672, 312, 68 + 1Kb + vector

Acc I should cut beyond 3' end
∴ 2164 - 3' + 5Kb

265-7
H3
H3/Bgl2
Acc I
H3/Nco



Hind 3/Nco
997, 1.7Kb, 312, 1Kb + vector

Only - Acc I cut sup this is correct

Where is the 300 bp frag

Addition of ERP-3 to 26S.

Within 5' 500 nucleotides of 26S unique sites are

~~Asp~~ Asp A1
BstE2 ✓
Aur 2
Sfi 1
Dpn M1
Nhe 1 – cuts ERP-3 twice
None cut the vector.

∴ How to insert

→ As before?
Sca 1 —— x

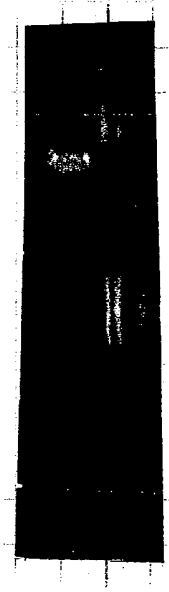
∴ Digest ERP-3 with remaining four enzymes.
Only BstE2 is available
→ ∴ Cut with it - Bug-
→ Cuts only once.

~~Cut 1st with Sca1 - High Salt - 37°C~~
~~Add 15u BstE2 - 60°C, then,~~

2-9-93
Cut 10ug E26S-Sal with BstE2 > cuts in Cgene
ERP-3 with BstE2
in medium salt
~~- Etoh ppt~~
- Digest with Sca1 in high salt.
Will not work — Sca1 site inside ERP-3.

∴ Check ERP-3 for E26S-Sal non cutters
① XbaI site – probably present
② Pst
③ Sac1
④ Sal1
⑤ Xba

From this point – will have to use Frags separate from vector.



2-15

ERP-3 digested w/ H3
pZ65-6 digested with XbaI

Both cuts complete
Frozen at -20°C

2-23

Phenol extract
EtOH precipitate

Digest both with BstEII
And separate on agarose gels

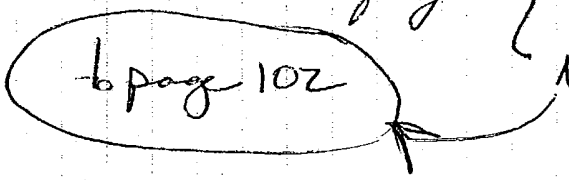
ERP-3 yielded a high MW fragment &
Agarose plug was frozen at -20C.
NOTE- FRAG WAS SLIGHTLY SMEARED WITH VECTOR
BAND WAS CLEAN.
Z65-6 didn't cut with BstE - only a single
band on the gel.
∴ Do not digest.

That's because Primer dor used to
generate the fragment starts at at 160 while
BstE 2 site is at 130

May require joining at Avr2 site if one
is not present in ERP-3

2 Avr2 sites in ERP sequence.

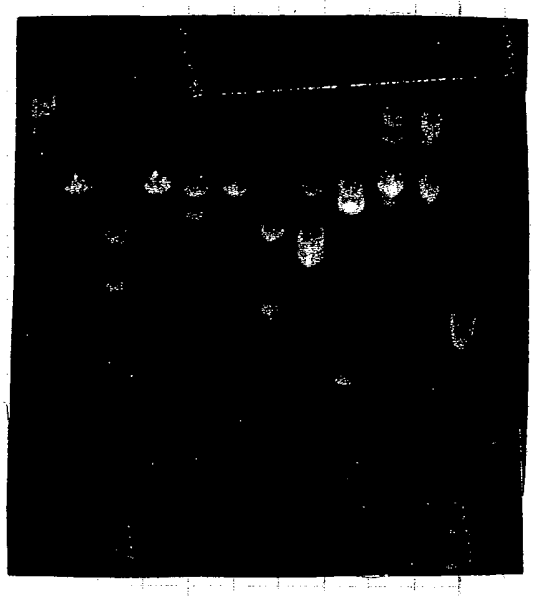
First Insert Nhe1 fragment into Z65-clone.



Assembly of 265

From pg. 98 2-7-93

Remap E265-5.2 & EEE-1



- linear
- vector 1.9KB
- linear EEE-1 +1.5KB (Incomplete)
- linear
- vector + 1.8

R1
R1/Xba
Xba
Bgl2/Xba
Bgl2
BAM

Vector + 2.6KB
+1.4KB E265-5.2 (pTZ18)
+1.4KB
linear

Sal1
R1
Xba/R1
Xba
pTZ Ddc

EEE-1 should also give 300 bp frag see on page 50 of book 3353 seen faintly in 4,5,6 on page 98.

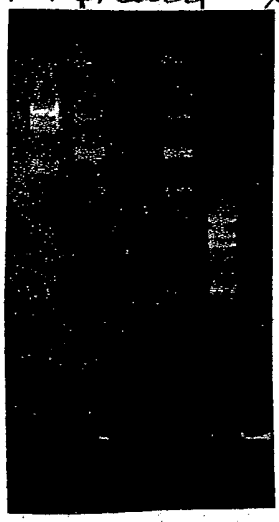
Redo Hind 3 digests of 4-7

#7 appears alright if 312 bp frag is visualized

2-7-93

Grow 200 ml of 265-6 & 265-7 O/N and continue mapping & terminal sequencing

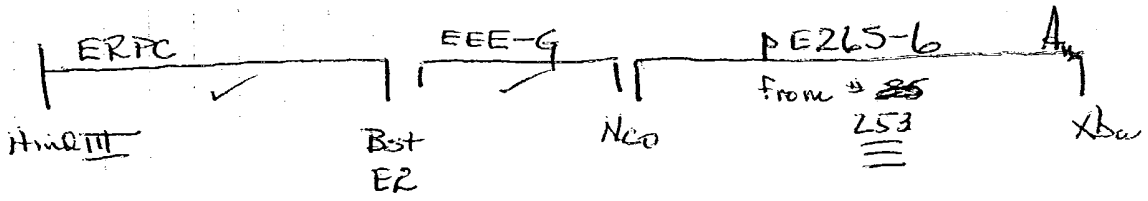
Frozen stocks in "Temporary Box".



after putting miniprep of 265-6 into Suic & JM105's again
Suic cells & JM105's, lanes 2 & 4 respectively
both yield proper H3 digests

Suic Culture #1 frozen in slot 253
E265-6-Suic had amplified to 200ml

From page 100



9-3-93

Modification of 3' end — See book 3552 pg 35

- EEE-1 put into CJ376
- Pick 3 colonies and make Φ
- Do SDM by standard T4 Protocol.

2-14-93

Deletion of 4183 MM

stock [DOA] = 589 ng/ml

3 µl + 5 µl 10x high salt
12 µl H_2O
3 µl RI

37°C, 1 hr 6:15

Also do RI/H3, Xba, Kpn, Bam cuts.

from page 94

Deletion of ERP-C

2-14-92

Based upon the PvuII digest

① Numerous small fragment should disappear first w/ Exo III treatment
See Restriction cuts of ERP-C and overlapped region of ERP

② The 1700 bp frag is the most resistant & the last to disappear

③ The ~ 400 bp frag disappears after the small frags.

∴ Insert size is predicted to be
7=10 > 8 > 12 > 1=2 > 6 > 5 > 4 > 3

Sequences are run for 7, 8, 12, 1, 6, 5, 4, 3

	locat'n of minus	
on gel	1 = 7	= 2-E
	2 = 8	2-F
	3 = 12	3-H
	4 = 1	1-D
	5 = 6	1-E
	6 = 5	1-H
	7 = 4	1-G
	8 = 3	1-F

Note that predictions are opposite what is expected based upon Exo III digestion time

→ Only 1-E was useful here. Stud frozen

Sequence to this point indicates that Cla I may be useful for further deletions

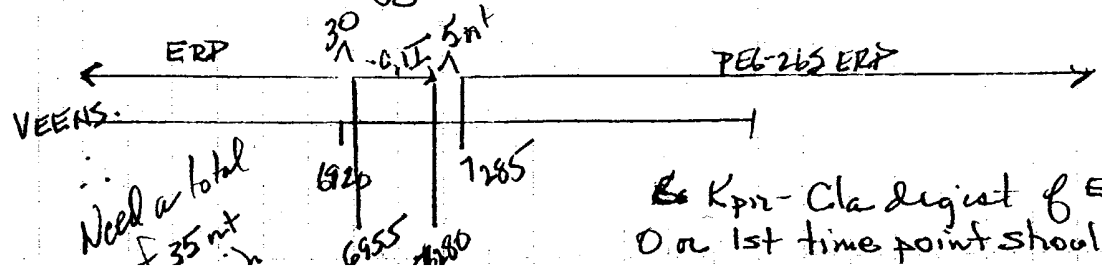
∴ Digest with Cla to check and prepare larger batch digested with Kpn, -Sac

Worked extremely well.

2-16-93 6 samples taken for ligation

2-23-93

Based on homology to VEE NS the map looks like this today



∴ Need a total of 35 nt in

& Kpn-Cla digest of ERP-C
0 or 1st time point should provide

extends to VEE NS 7280

2-25-93

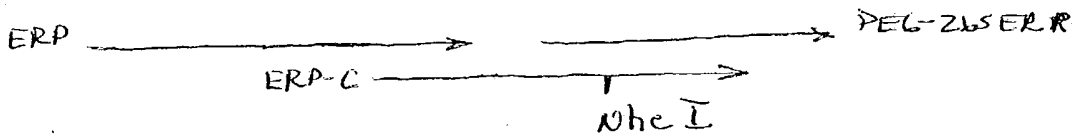
As predicted, Kpn-Cla digestion removed the 5 nt gap.

Gap between ERP & PEG-26SERP still remains

2-26-93

∴ To get at it

~~Digest ERP-C with~~



Double digest in M buffer

① Digest ERPC with Nhe to ~~drop out~~ open the plasmid.
② Digest with Hind3 to remove downstream sequence, AGE, gene clean
③ Repair & ligate
④ Sequence with forward primer to FILL gap.

Done 3-4-93
Frozen in sequence box at -70°C

Cloning 5' end of PEB from page 20

E CDOA religated to pTZ19 HindIII - O/N room T

Into HB101

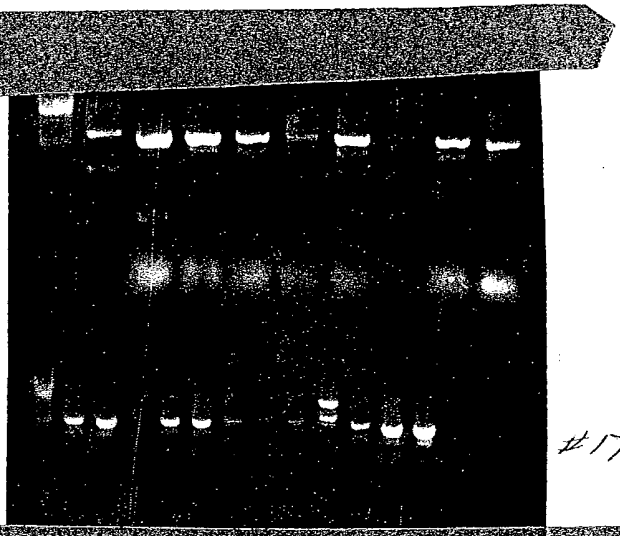
3-1-93 Discarded, vector background too high
Prepare new vectors both pTZ 18 & 19.

After digestion
Add 2.5µl 20x CIP + Mg Buffer
3µl CIP
37°C, -30'
Phenol
EtOH
OD_{260} 0.267
Ligate E CDOA.

Sample	Insert	Vector	10x	H_2O	Lig
Vector	—	2	3	23	2
EEE	5	2	3	18	2
OEE	5	2	3	18	2
	✓	✓	✓	✓	✓
		4	12	72	

3-4-93
Prepare R1/H3 from ERD-7 for probe

3-5-93
Prepare probe
1µl DOA
1µl random primer
12µl H_2O
DH
5µl ^{32}P dCTP
5µl dart dCTP
5µl 10x Mcl
1µl Klenow
37°C 30'
5µl EDTA
Spun Column
µl cpm - 270K
O/N 69°C 2hr wash



#13 Cuts
EP-7, 1-8

9-20

#17 ≠ EEA-3 by Comparative Maps.

to pg 117

5 end cloning Repeat.

Standard CDNA synthesis with Randomm priming ± ^3H -Labeling

	EEE-	WEE - B-11 RNA 10ng/ml
	E RNA ✓	✓
20µl RNA in H_2O	✓	✓
42°C - 2'	✓	✓
3µl random primer	✓	10 c olings dT
10µl dNTP 5mM	✓	1-dT 14-20
4µl BRL RNAsin Promega	-	Pharmacia
32µl buffer cocktail	✓	1mg/ml
2µl H_2O	✓	185 25-50 bp mobile
2.5 µl RT HME 357-1 100-140K/µl	✓	
3µl dGTP - ^3H	✓	
5min RmT / 42°C, 1h		

2µl samples.

→ Too little isotope here for useful numbers

+ 8µl EDTA
+ 15µl 2M NaOH
70° stopped 37°C, 5.10 - 6:00
- 5µl column
- 100µl TE
- Phenol 2x
EtOH

+ 8µl EDTA
92µl TE
Phenol 2x
$CHCl_3$ 1x
133µl $2M NH_4OAc$
800µl EtOH
↓ -70C ↑ RmT
Repeat

to 111

3-1-93

It appears that there is a mistake in the ERP sequence i.e. there is a series of termination codons between nt 1300 & 1500.

∴ Do a series of deletions
1) Is the Bgl I site at 1290 unique
↳ no other cuts vector.

2) XhoI ? – ~~no~~ cuts vector
3) ~~Sma I~~ ? OK (332)
4) ~~Sst 2~~ – no vector cut (1494)

One termination in ERP-7

ERP-7A1	~40	Frames in -2	pushes up hydrology
ERP-7F	← ~60	↓AA	45-60
-7,2A	~83		135-200

7,7A

3-6-93
Corrected from 7,2A & 7A1

3-7-93
Examination of ERP & PE6 Genome Sequence.

Translate of ERP –
-3 areas 1 – ~ 80 AA
– 80-325 numerous stop.
-2 areas 325 – 750 (end)

11-1
12-1
Direct

cDDA cleaning cont'd

from 108

WEE

Label w/ 35 in second strand.

1µl H_2O
25µl cocktail
3µl 35
1µl ROX-II
2µl PCI

1µl samples

Repeat of Eb cDNA cloning. 3-20-93

It appears that ERP-12 may be a DE, redo the cloning. Stock RDA ~ 300ug/ml

#1- E vRNA 18ug/ml 10ug of each ethanol precipitated

#4- Pure RDA - 12-2-92 Pellets look clean, light brown
└ 280ug/ml

50µl RDA precipitated

BRL Random Primers.

follow protocol on page 43 Promega RDA kit

3µl RT ~~AMV RT~~ HME-357-1 Lot Lucigen

~~4 units~~ LME 704

- 8:10 AMV RT → Lot L51819XL

2µl samples 1, 2, 3 - V

5µl EDTA 4, 5, 6 pink 12-2-92

~~5µl~~ ~~Agarose gel~~

	Sample	CPM	Net	%	nM	ug cDNA	Purified
100µl TE							
Ø-OH 2x	1	565271					
CHCl3 1x	2	2793					
133µl NH4OAc	3	14647	16854	0.248	5.96	1.96 µg	
1ml EtOH							
↓-70 1 hr		453842.5					
Spin	5	1932					
Rinse	6	13477	15415	0.041	4.7	1.39 ug	
Dry.							

2nd strand Synthesis

17µl H_2O 22µl ✓

25µl cocktail ✓

5µl 32-dCTP diluted —

1µl RDAseH ✓

2µl Pol I

12°C - 1hr 9:30 - 10:30

22°C - 1hr 10:30 - 11:30

5µl EDTA

148µl TE

Ø-OH

20µl NH_4OAc

500µl EtOH, -O/N.

Pellet: Dry

(over)

3-4-93

Spun column

count/µl each	V	90	11,100 cpm
	12-2	98µl	6300 cpm

Volume of		µl dATP 5mM	vol 10x 74 Pol	74 Pol.
V	89	5	10.5	1
12-2	97	5	11.3	1

37°C - 30'
4µl EDTA
5µl to ABE - vertical Master V 12-2
Phenol-1x
~~Spun column~~
EtOH

EcoRI linker ligation - Both Samples
23.5µl H_2O
3µl 10x Ligase
1.5µl 100µM EcoRI Linker - NEB
2µl DDA ligase
1µl (200U) NEB DDA Ligase
Rm T-O/N.

ΔH - 10'
6µl 10x High
50 Units - EcoRI - 5µl
q.s. 60µl
37°C 2hrs 7:40 a.m. - 9:40
fractionate ligate.
Electroelute.
3 FRACTIONS FROM EA IV LARGE TO SMALL

V-1	A	12-2	4
V-2	B		56
V-3	C		

EtOH ppt'd
Rinsed
Dried
Ligated

to page 119

3-15-93

Determination of small gap in pE6-26S RDA.
and in PE6 2S. - pg 116

Based upon sequence, an Eco RV site should be present at ~ 3833.

Using pEEE-1 (Born Site of pSZ19) - Book 3353, page 50
- Digest 3mg w/ RV
- Check digest on gel - should also release ~ 500 bp frag.
- Digest with Eco R1
- Blunt end
- Purify
- Ligate
- Sequence with forward primer

It appears that one of the RV sites is missing from EEE-1
∴ Digest 1mg of RV cut with Xba! ?
How big is the release fragment?
~ 600 bp

Reread gels
Site at 3497 is definitely there.
Could be some ambiguity & other site due to -CGCG-
gatatc is faint on gel.

RV/Xba digestion release ~ 600 bp fragment.
∴ 2nd RV site at 3833 must not be there.
∴ Re sequence EEE3B with (EEE-E11) PCR primer
Using dITP tubes - sequence EEE-E1 EEE-1, include Ø as control. (plasmids)

4-21-93
Three primers representing 3' portion of genome
EEE3'
EEE3'52'
EEE3'INTERIOR - Being made 4-21-93
Use to sequence on EEE-1 template

5-15-93

Two primers designed based upon ERP7 & ERP-3 sequences to PCR the gap between the two primers ERP7-A & -7B phosphorylated and used as usual.

492-5B of genome 1-4-93

Fragment = 700 bp blunt ended with T4 Polymerase, purified by Alt and ligated into pTZ18 Hind 2 Tube 35

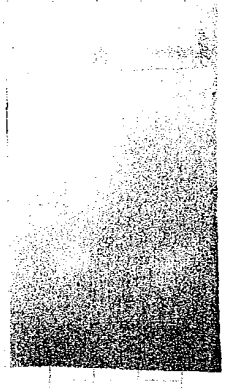
5-16-93 O/N.

Put into 5 ml cells and plated on X-gal plates.

Remaining fragment stored in Slot 46 -20°C.

ERP7 PCR

Vector	2 ✓
Insert	5 ✓
10x	3 ✓
H_2O	19 ✓
Lig	1



= eluted and ligated pTZ18 Hind 2

5-19-93

Twelve white colonies picked and R1/H3 digested

#7 picked for sequencing of plasmid.

Also into 5 ml cells

FROZEN -70 Slot 248

ERP-7 PCR

Comparison of #7 Insert to purified PCR frag.

Sequencing done P.M. 5-19-93

Sequence verified as predicted

Cloned in negative sense w/respect to T-7 promoter

Sty 1 Kpn 1

5/20/93 DNA stock in Slot 44

Sty site ?

Dig cut with Kpn 1 in 20µl — internal site ?

Characterization of clone ERP-17 3-20-93

Southern blot.

Digest ERP-17 & ERP-3 with - EcoRI/HindIII, ^{32}P-labeled marker
- HincII
- Sty I

- Semidry blot to Hybond-N
0.8 mA/cm^2
90'
- UV irradiate dried blot 2.5 min
- Prehybridize w/ BRL Perhyb Buffer ←
- Probe - ERP-7 frag
10 µl H_2O
5 µl DOA
1 µl Random primer
STH, 10'
0°C.
5 µl dATP-dA
5 µl ^{32}P-dATP
5 µl 10x Mod Sect Buffer
7 µl H_2O
1 µl Klenow
Room T - 60'
5 µl EDTA
45 µl H_2O
Spun Column 1 µl eluate 59K cpm.
not Vacuseq blot

Perhyb
10 ml American Hyb Diffco
+ Probe - 3:35 p.m. - 6:30 3-20-93

No detectable hybridization. Control is positive - autorad.

From 114

Cloning PEB into pT219 EcoR1

Vector pT219-EcoR1 8.3ug/ml tube 21
Entire eluted sample used

Sample	H_2O	Vector	10x	Lig
A (15+6)	21	4	3	2
B	21	4	3	2
C	21	4	3	2
4	21	4	3	2
5	21	4	3	2
6	21	4	3	2
Vector	~~30~~	4	~~4~~	2

Remainder frozen in -20° BX

Pellets dissolved in 15µl H_2O
4' 65°C, 5'
Cocktail of remaining reagents added
RmT – O/N.
Dil to ~~1:5~~ : ~~onto xgal plates~~.
HB101 on AMP plates. AM. 3-23-93
Each tube suspended in 15µl - duplicates were eluted - and combined
∴ 30µl H_2O
+ ~~5~~µl 10x Lig + 4µl Vector + 2µl Ligase

Numerous colonies on all plates - vector included.
Background w/ vector previously determined in JM105 - ∴ Not anticipated in HB101

- All plates lifted - few/any growth on plates.
- Prehybridized at 65°C w/ BRL Rediprb Buffer,

Probe preparation
~~1~~ 22µl ~~V~~ RNA preparation
3µl Random primers
65°C – 2 min
5µl ~~32~~ 33P
5µl dNTP-dA
3µl cocktail
5µl Promega DNAse
27µl H_2O
3µl RT
5' RmT 42°C – 1hr.

Discarded

1µl = 470K cpm

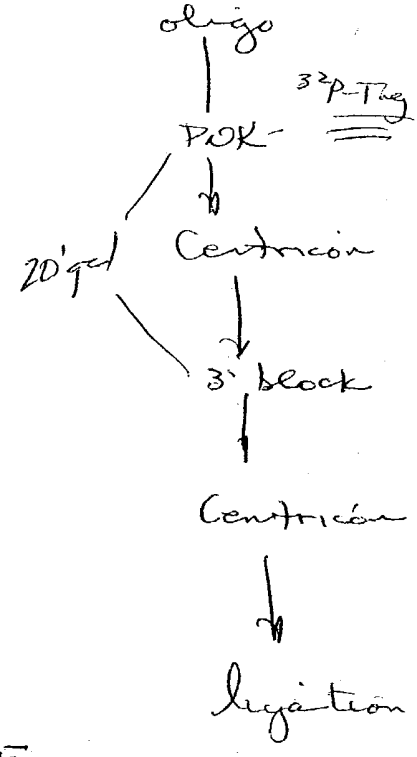
Ads blot 8:00 p.m.

3-23-93

Preparation of oligo for SLIC

46mer detritylated purified.

500ng = 33nM



PNK- 50µl rxn
5µl 10x
5µl 10mM ATP = 1mM
5µl γ ATP
Spermidine 1mM.
9.5. 4µl
1µl PNK

1hr 5µl EDTA
DH - 10'
H_2O to 1ml

Centricon-3 → 50µl → 2µl for PAGE
20µl 5x TdT
20µl 5mM ddATP
10µl H_2O
2µl TdT
37°C - 1hr.
5µl EDTA

PNK reaction
500ng oligo 86ng/ml = 86ng/µl
6µl oligo
+ 5µl γ ATP
5µl 10x PNK
21 5µl 10mM Spermidine
29µl H_2O
2µl PNK
15' 37°C 55°C 3.10
Bring ATP to 1mM 5µl 10mM
30 45' 37°C.

5µl EDTA, 65°C, 10'
Centricon 3
Dilute to 1ml w/ TE

3-25 from 121

Significant counts remaining in ~ 50µl

3' blocking

✓ 20µl TdT Buffer - (BRL)
20µl 5mM ddTTP
q.s. 98µl — 2µl sample PNK—
2µl TdT - 7:10 (GIBCO-BRL)
37°C, 1hr
5µl EDTA
65°C, 10'
— 2µl sample PNKT

When run on 15% sequencing gel, no evidence of band shift.

Redo with my own TdT Buffer.
Have not homemade - only BRL

As control
Use TdT to add dCTP to oligo & look for laddering.

1) Run a portion of labeled oligo through spun column - Does it flow through?
10µl of oligo - 32P
90µl H_2O — count 5µl - 29000
column
→ 5µl count - 9300

The initial filter concentration would not remove much of the P oop.
∴ The final counts are reasonable for efficient labeling & clean up.

∴ Run the remainder through spun Column & proceed with TdT assay as before.
2µl = 17K cpm.

1

3-31-93

Blocking 3'end - pilot repeat

5μl 5x TdT Buffer ✓
5μl 5mM dcATP ✓
10μl 8 labeled oligo ✓
3μl H_2O ✓

└ 10μl sample ✓ TdT-0

1μl TdT ✓
37°C, 1hr 10:35-11:35

└ 10μl sample
TO 15% gel.

4-1-93
Reaction is positive.
∴ Scale up for primer preparation.

Adjust remaining oligo to contain buffer components as described above.
Before adding TdT, take 2μl sample.
Label TdT-30. Store at -20°C

Vol of labeled oligo = 9μl
∴ Set up 150μl reaction
30μl 5x
20μl 5mM dATP ✓ 5μl sample
5μl TdT ✓
6μl H_2O ✓
@ = 11:35

6μl sample

Sample 6 5% seq gel

Preparation of cDNA
Standard RT reaction
✓20μl RNA 10 ug - vRNA 5μl EtOH ppt.
<2°C - 2! 2μl sample
Ortho ice
✓ 3μl random ✓ ✓4μl ^{35}S
✓ 10μl 5mM dNTP ✓24μl H_2O
✓ 3μl ~~Random~~ RNAsin 4μl RT 5"RmT
42°C, 1hr. 3:25 ~~4:15~~ 4:15

~~Prep~~

t_i - 634461
t_o + 1186
t_f 7873 665 7 ~ 600ng cDDA

Low Mt Synthesis

EDTA to 20 mM 5µl
NaOH to 100mM 2µl 10M NaOH -
37°C - 9D.

Preparation cDDA for SLIC

- Neutralize cDDA
- Phenol Extract 2x EtOH
- Spun column into H_2O
- Adjust to Polaxe Buffer 1µl = 203cpm
  Terminated

Repeat RT

Preparation of CBA87 WEE VIRUS. 4-3-93

1) 4 vials of CBA 87 Stock diluted to 10ml
2) 10 rollers inoculated with 1ml
3) Refed with 5% FBS, P/S EMEM
4) Rolled for additional 24 hrs.

4-4-93
CPE becoming evident.
∴ Remove media as inoculum
in 1ml aliquots from rollers.
Labeled WEE CBA 87
SMB, 2 PASSES IN BHK
4-3-93

First lysate onto 1 gradient. Wash buffer into second.

- Scrape cells into 20ml cold PBS.
- Pellet 2K, 10 min.
- Suspend in 2ml TKM NP40
- Vortex vigorously
- Pellet 1 min
- Layer onto 35% CsCl
- Spin 90. 33°K, 20', 18 hrs preferably

Questions
1) is nucleocapsid band visible? - No!
2) visible RDA pellet?
Heavy pellet seen!

Virus prepared from Rollers of BHK cells
RDA prepared by phenol extraction aqueous
contaminated by some brown pigment.

Use 5ul in RT: oligo-dT primed reaction
RT-5ug oligo-dT 5ug.

Sample	T_0	T_{0+}	T_F	net	% nucleus	in ug
RD	105450	2642	23586	20944	0.039	5.8	1.8 ug
oligo	90585	2112	10009	7897	0.0059	1.74	0.54 ug.

Inadvertently held at 65°C for 2½ hrs.
Freeze at -20°C o/n for PCR.

Start PCR 4-15-94

PE6 - cDDA repeat

4-17-93

Still can't find DS clones.

Note that H_2O bath originally at 44°C.

- 2µl RDA in H_2O — one tube protocol 2µl ~~ethyl~~
  4/2°C - 2'
- 5µl random prime - 1mg/ml
- 10µl d-NTP
- 5µl RDA sin
- 32µl Buffer cocktail w/KCl
- H_2O 2µl
- 3µl ^{32}PdCTP
- 4µl RT
  2mT-5' 42°C - one hour
  2µl samples. 0', 0+', 60+-

$t_{\frac{1}{2}}$	509910	
+ +	1248	
0	25931	3.015ng
60	48700	5816 ug
120		↳ Extremely High.

Add To remaining 60µl EDTA to 200µl

To 40µl add — To remaining 60µl EDTA to 200µl

125µl (150) 40mM tris pH 7.5 ✓ — 140µl H_2O

(100) 10mM $MgCl_2$ ✓ — Phenol 2x

(200) 20mM $(NH_4)_2SO_4$ ✓ — 133µl 5M NH_4OAc

(700) 200mM KCl ✓ — 800µl EtOH

(100) 100ug/ml BSA — -70°C.

4.6 Units RNase H

115 Units Pol — Add additional dNTP - 2.5µl to bring in line w/ Gubler protocol.

12°C - 60'

22°C - 60' - 5µl sample

ΔH - 70°C, 10'

15 Units T4 pol

37°C - 10'

SDS to 1%

Phenol extract

Ethanol ppt.

→ Aborted here because RDAsin was added to first strand buffer.

Additional 1µl fXL AMV RT added at one hour

Continue for added 1 hour

EDTA, Phenol to 2M NH_4OAc, 800µl EtOH

frozen to -70 O/N

93 Warmed to Rmt

Spun

Washed

4-18-98

2nd strand Synthesis
22µl H_2O
5µl second st buffer
1µl RNAse H - BM cDNA Kit
3 ~~5~~ µl Pol I BM cDNA Kit
~~8 5µl~~
12°C, 1hr ✓
22°C, 1hr

Heat Inactivate - 10min, 65°C.
20µl dH2O
Add 20µl 10x T4 Pol
10µl H_2O
2µl T4 pol

37°C, 20'

8µl EDTA
Phenol 2x
~~20µl 5M NH4OAc~~
~~100µl EtOH~~

~~Pellet, rinse dry~~

Spun Columns.
Count 1µl 1µl 22K = cpm

Combine
Add 20µl 5M NH4OAc
600µl EtOH ~~Stopped~~ 4-18 5:00
Pelled, rinse 1 dry.

- Run half on agarose gel
- Cut 8x fractions
- Electroelute + ppt

Ligation - H2 vector

	Sample	Vector	H_2O	10x	lig
	1	2	3	2	2
pT2-18 Hind 2	2	3	3	2	2
13ng/µl	3	3	3	2	2
	4	3	3	2	2
Tube 35	5	3	3	2	2

Dig 3P

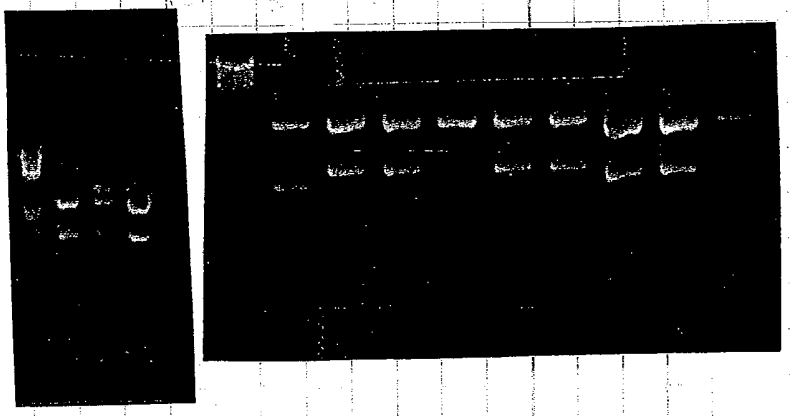
from 74

CV gap PCR continued

4-19

12 colonies picked and digested with EcoRI + HindIII

Most of the cuts contain an additional ~1Kb frag in addition to the expected digestion products.



Sequence 1 + 7 from both sides (puc13)

#1 gives sequence equivalent to 3' end of SFV as shown + evidence of 3' oligo-dT tail.

Sequence file of 80nt saved as CHK3'
O/N maxi started to continue the sequence

Freeze in slot 265.
- Actually EEE3' #1 identical to EEE1.?
- Discarded

CV gap continued

- CHK3' and CV gap 1 used in standard PCR.
- Band present ~ 700 nt at
- T4 repaired
- Phenol, spun column
- PCR
- gel resolved
- Ligated to PTZ18 Hind 2
- Into Sure cells
- On 6 x gal plate

See book 3526 page 69.

7-24-93

PREPARATION OF ADDITIONAL CHIK PARENT vRNA

VIRUS

15561 AG #1095

- 2 OF ORIGIDAL

24 AUG 1978 FROZEN in SLOT 69, BOX 2

0.5 ml diluted to 10ml

2.5 ml into 4 rollers of BHK cells

ADD EMEM NEAA + 5% FBS + ERS

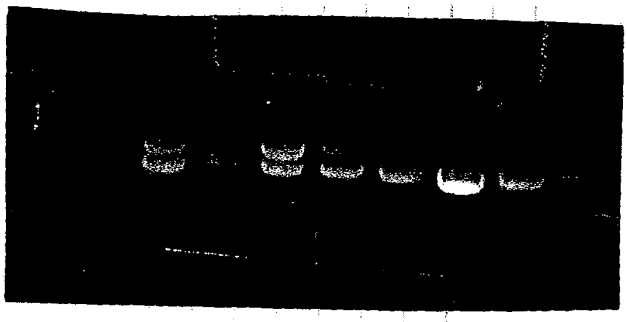
from page 128.

4-27-93

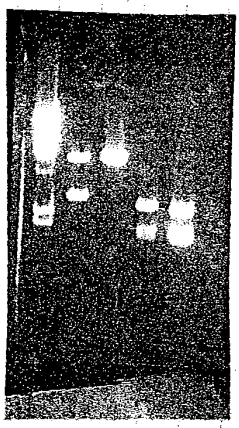
9 positive clones picked & digested with R1 + H3

#2 + #4 appear to have inserts.

To make sure: digest each with Hind3 & Pvu 2



1 Hind3
2, 4 Hind3
2, 4 Pvu 2



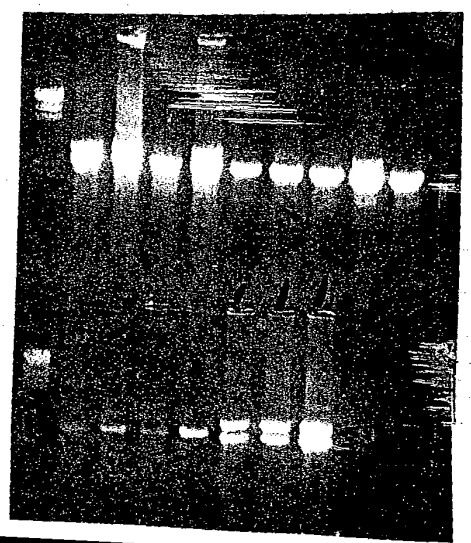
SEQUENCE OF 4 4-29-
CONTAINED WITHIN ERP-3
DISCARDED

2
NOVEL
DISCARDED

5-1-93
26 MORE COLONIES PICKED AFTER VCDDA PROBE

- SHAVE INSERTS TO SAME SIZE
- DIGEST WITH PST I

R1/H3



λ

Xhoi cut
Apa I EcoR I Bcl I
Kpn I Sa I

6-5-93

Replicon Helper Preparation.

272-3 (or is it 272-3) Repeat of Xhowt 5-11-93

E276 lys, E2109 lys, E1272 thru

① Digested with Apa I + Kpn I ✓ 7505 L 955, 3357 → deleted ~6KB

② Blunt end with T4 PL 2µl 10x 1µl dNTP 16µl H_2O 1µl T4/PL

③ Purify and electroelute ~7KB band

④ Ligate

⑤ Transform JM105

⑥ Picked 10 colonies

⑦ Digest with R1/H3 with 272-3 control

previously determined map is missing R1/H3 predicted to not cut deleted form.

Identify clone with ~5KB insert + 2KB vector

Predicted VEE sequence file VEE/A&A/KDW

SELECT # 4 FROM FIGURE FREEZE IN SLOT 250

~~Remove~~ 272-A/K ~~PRIOR TO~~

⑧ TRANSPLANT R1/H3? FRAGMENT INTO PHAGEMID IN POSITIVE ORIENTATION.

Into p72B + 19. Restriction map of clone not yet complete.

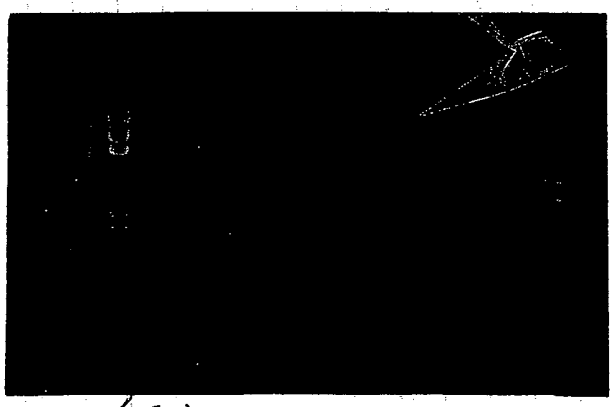
Step 7

KB

λ H3

1-10

272-3 Control.



Ligation	Vector	µl	Insert	H_2O	Ligase	
18-	1.5	3	5	18	2	The ligation
19-	2 ✓	3 ✓	5 ✓	16 ✓	✓	

Into Sure cells 5-7-93 p.m.

✓ X X

Based upon restriction map the pT2 19 recombinant

5-10-93

All six clones contain predicted fragment.
Save #9 Slot 239 ~~272-3d-19~~ Discarded
Replaced with A
Put 1.5µl of mini into CJ236 cells. #'s 1-3 Pg 9 Book 3516
Many colonies freeze in slot 240 CJ 272-3d19
- 1 picked from each plate
- Grow up
- Start 20ml ϕ prep.
Harvested and purified
33ml PEG/NaCl + 20ml ~~purified~~ clarified supernatant

Need to prove that vector is pT219 and not recombined parent vector.
∴ Do comparative R1/H3 Cut. 5-15-93
Digest #'s 1-3 and compare with 272.3
Do vector comigrate
No! true pT219?

5-18-93

Preparation of phosphorylated Rep-1 oligo

Oligo prep at 126ng/ml MW = 42 x 312
Slot R-2 9.6µM 13104

25µl oligo
6.3µl 10x PNK
6.3µl 10mM ATP
23.4µl H_2O
2µl PNK
37°C 1hr 3:45 - 4:45
65°C 20'
freeze at -20 slot R-2

5-19-93
Redo with both on same gel
272-3d19 / R1/H3 digest
272-3

5-22
~~Digest with H3~~
Mini's do not appear as the mini's do
5-23 ∴ Redo mini of 19-3 my magic prep
Cut 7µl w/ R1/H3

5-12-93

Site directed mutagenesis

Suspend dut-ung- ØDNA in 5µl H2O

10ul template
ul 160ng phosphorylated Rep1
75 9.5 18.75µl
1.25µl 20X SSC

DH 70°C 10'
Cool to room T.
5µl 5mM dNTP
10
10ul 5X T4 buffer
9.5 95µl → 60µl H2O ✓
1µl ~5u T4 Pol ✓
5µl ~5u Ligase ✓

Used standard buffer here

5X
100mM tris pH8.8
10mM DTT
50mM MgCl2
2.5mM dNTP
4mM ATP

Room T - 10'
37°C - 2hours 4:30-6:30 ✓
EDTA to 25mM ✓
Transform JM105 ✓
1/0 colonies

5-14-93 Redo transformation onto DH5α
~8 colonies picked for minipreps

5-18-
AGE indicate no Ø DNA in preparation
∴ Redo.

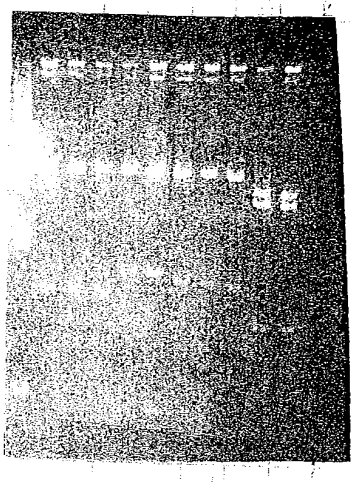
5-23
Repeated preparation of plasmid shows that
maxi preps fail to yield desired product.
∴ Redo the ligation & transformation.

5-24-93, mini's 19-2 & 19-3 retransformed into
105's.
→ Pick 5 colonies of each for mini's ← Nco1 3,4,5,6,9
Aflll 1,2,7,8,10
labeled, dl-10 [illegible]

Because subcloned products are not as expected
Do AvaI ÷ NcoI digest of 250 - 272-3 ApaI/KpnI
is this as predicted.

Subclones 3,4,5,8,9

3,4,5,8,9 AvaI
NcoI



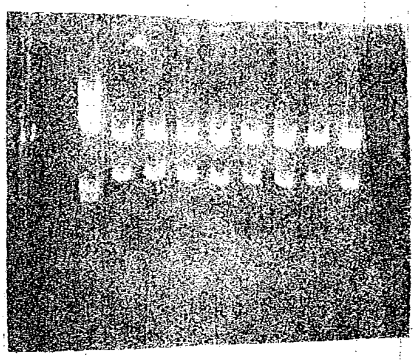
No equivalent digest on #8
Colonies picked from second ligation
A—H

Start over here of ApaI/KpnI deleted 272-3
① Check predicted restriction maps
② Repeat entire segment.
Also check KpnI digestion of
Also cut 5uL purified frag with AvaI
÷ NcoI
~~Previously purified material of 5-5~~

AvaI of 272-ApaI/KpnI #28 = 1.3 Kb frag
NcoI " = 2 Kb

5-27-93

Eight additional clones from
2nd ligation
RI/H3 digest
Insert seems a little
small.
Do AvaI and NcoI digests.
ApaI
? RI/H3 > Compare with
parent 272-3.



To Book 3516
pg 9

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.